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# THE STREPTOCOCCI

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When one attempts a review of the streptococci, within reasonable limits, he must at once proscribe many interesting and important phases of the subject and thus delimit the field of discussion. At the outset, therefore, it should be understood that the matter to be considered is that which appears to contribute to a better understanding of the relationships within the genus *Streptococcus*; the units or component parts being those somewhat homogeneous groups, types, or species which make up the whole; a "species," necessarily a nebulous concept when applied to bacteria, being defined along somewhat time-honored lines. Whatever may be the proper species concept with respect to streptococci, it is probably safe to assume that the lines of demarcation fall somewhere between the ultra-conservative "*Streptococcus hemolyticus*" and "*Streptococcus viridans*" stage, and the hair-splitting divisions which have been set up by some taxonomically-minded bacteriologists.

So limited, the scope of the proposed discussion becomes somewhat more clear: The technical importances of the streptococci as agents of disease and in industry are, except incidentally, beside the point; the voluminous literature on the nutrition, growth, and general physiology of the streptococci cannot be considered, except for those phases which contribute to a systematic synthesis; serological methods which cut group or species lines are included as tools, but the immunological aspects of the streptococci are not reviewed. Nor is variation, as such, considered; this phase of the streptococcus problem, however, has been included in the comprehensive reviews by Hadley (1927, 1937).

Without entering into an elementary definition of the genus

*Streptococcus*, it may be noted that this review is limited to forms which are bile-insoluble; which do not produce carbon dioxide, nor large amounts of volatile acids and other volatile compounds, from fermentable carbohydrates; which produce lactic acid, principally or exclusively of the dextro-rotatory form, as the dominant product of carbohydrate fermentation; and which are facultative with respect to oxygen requirements. By definition, this eliminates the members of the genus *Leuconostoc*, the pneumococci, and the anaerobic streptococci. The exclusion of the latter may or may not be justified in the light of present knowledge: it is probable that some true streptococci, in a physiologic sense, are anaerobic; but those anaerobes which produce gas and putrefactive odors would appear likely to prove as distinct physiologically from the streptococci as are the pneumococci or the members of the genus *Leuconostoc*.

As this introduction is written after the completion of the body of the text, it is quite simple to predict the outcome. As much material as possible is put in tabular form and this is supplemented with additional descriptive matter, more or less pertinent from the standpoint of taxonomy, in the discussions of the individual groups or species. It is of course impossible to include more than a very small fraction of the literature on streptococci, even though limited to those papers which bear, in one way or another, upon the taxonomic aspects. The references actually cited are those which seemed to have pertinence as the text developed; but in addition to an incomplete bibliography the choice of selections could doubtless be improved. But one point should be kept especially in mind: were the selection of citations faultless for present purposes, much of the most distinguished and important work on the streptococci would not be touched. As a review, not a review of reviews, this paper makes no attempt to discuss the many admirable classifications of the streptococci which have been made in the past.

Attention has not been given to the numerous streptococci which have been named as "new" species without adequate descriptions to differentiate them unequivocally from other types. On the other hand, it should be recognized that the

species which are now fairly clearly defined represent only a portion, perhaps a very small fraction, of those which actually exist. In the case of the hemolytic streptococci, the findings with the Lancefield serological technique, which have already brought several new groups to light, point strongly toward such a view. Nearly all workers with this method have reported a few "unidentified" hemolytic streptococci—in most cases probably strains of unknown species of relatively rare occurrence in the sources commonly explored by bacteriologists. For the most part, the known species of streptococci are those which have brought themselves clearly to our attention as the agents of disease or as more or less dominant organisms in familiar habitats.

#### METHODS

Progress in the identification and classification of streptococci has been made only as improved methods have been brought to bear on these problems. Although a detailed consideration of methods might appear appropriate, space does not permit more than a mere mention of the tests employed, with citations, so far as possible, of the originators of the various techniques. The tests used in the tables of this review are those which have had wide enough application to the various groups of streptococci so that their general significance is reasonably clear. Other special methods which have value in connection with certain types are mentioned in the text. It is doubtless true that many valuable methods have been suggested for the study of the streptococci, which have not been further exploited, but this phase of the subject is quite outside of the scope of the present discussion.

The original use of blood agar for determining hemolytic properties among the streptococci is of course to be credited to Schottmüller (1903), and Gordon (1905a, 1905b) was certainly the first to apply the fermentation tests in a broad and systematic way. In the case of some old but useful methods such as the liquefaction of gelatin, action on milk, and reducing properties, it is not possible to give credit to specific workers,

as these tests had long been in use for studying bacteria and their application to the streptococci was a gradual development and somewhat a matter of course. It should be remembered that the ability to curdle milk and to reduce neutral red were among the original "Gordon's tests," these two reactions being chosen by Gordon along with certain fermentation tests as having especial value for the study of streptococci. Although little employed in recent years, the ability to coagulate milk was used by all the older students of the streptococci and was considered by them to have distinct value as a differential test. In some cases the action of streptococci on milk gives information of at least supplementary value; in the case of the lactose-fermenting streptococci, the information obtained by this simple test has much the same value as the determination of the final pH in glucose broth cultures. As the use of litmus milk in the study of bacteria is very old, it is impossible to tell just when use was first made of reducing action in the differentiation of streptococci, but it is certain that the value of such tests was recognized before Gordon's systematic application of neutral red. MacCallum and Hastings (1899) described in detail the strong reducing action of the organism which now goes under the name of *Streptococcus zymogenes*, as revealed by its ability to reduce litmus in milk cultures before the milk was curdled, and through the years strong reducing action has been looked upon as an especially marked characteristic of the lactic-acid streptococci, and, to a slightly lesser degree, of the enterococci. Tests for the reducing ability of streptococci have considerable utility, and this value could probably be increased with some improvements in technique and the introduction of additional simple tests for this purpose. Litmus milk as a test of reducing ability of streptococci has been applied very loosely by most workers, no consideration being given to whether the litmus is reduced before or after the milk is curdled, whereas a few workers have insisted that the vital point was whether or not reduction preceded curdling. Recent studies have shown that the reduction of litmus in milk cultures before curdling is correlated with a low reduction potential, whereas the reduction which takes

place after curdling appears to have little significance inasmuch as such reduction may largely be the result of the action of the milk itself when convection currents are stopped by the formation of the curd (Knaysi and Sherman, 1937).

In the tabular material used in this paper, action on blood is limited to the true beta hemolysis as revealed in blood agar and to "non-hemolysis," including the various gradations from the gamma to the alpha reaction. Brown (1919, 1937) has accurately developed the use of blood agar in the study of streptococci and has shown the value of greater refinement in its application, but in considering the streptococci as a whole it is difficult, if not impossible at the present time, to draw accurate lines between the species of non-hemolytic streptococci based upon their action on blood agar. In such species as *Streptococcus salivarius*, *Streptococcus bovis*, *Streptococcus lactis* and *Streptococcus fecalis* different strains are well known to give reactions ranging from the gamma to the typical alpha type; and when consideration is given to such observations as those which have been made by Clawson (1920), Oppenheim (1920), Hagan (1925) and Gordon (1933), such variation among diverse strains of the same species would seem entirely logical. No attempt is made to use the so-called "soluble hemolysin" test. Todd (1934) has shown that with the application of appropriate methods all of the hemolytic streptococci studied by him produced soluble hemolysin. Todd tested representatives of the Lancefield groups A, B, C, D and E, and Long and Bliss (1937a) have extended these observations to members of other Lancefield groups. Hare and Maxted (1935) have called attention to the fact that, when tested by the conventional methods, the term "pseudohemolytic streptococci" may be applied with equal justice to some strains of several of the various hemolytic species.

A number of valuable methods for the study of streptococci were introduced by Ayers and his several able associates. The thermal resistance of streptococci was systematically studied by Ayers and Johnson (1914) in connection with the ability of certain types to survive the pasteurization of milk, and heat tolerance was later utilized as a significant physiological char-



acteristic in the differentiation of streptococci (Ayers, Johnson and Davis, 1918). Orla-Jensen (1919) applied thermal death point studies to a large number of types of streptococci in his investigation of the lactic-acid bacteria, and Houston and McCloy (1916) and Dible (1921) made use of thermal resistance as an especial characteristic of the enterococci. The final pH attained in glucose broth cultures (Ayers, 1916; Ayers, Johnson and Davis, 1918) has proved of substantial merit and has been widely applied by workers on the streptococci. The ability of certain streptococci to hydrolyze sodium hippurate was discovered by Ayers and Rupp (1922). The application of this reaction, for the specific purpose for which it was recommended by them, has proved to be one of the outstanding physiological tests which have been used in the differentiation of streptococci. The production of ammonia from 4 per cent peptone by streptococci was recommended by Ayers, Rupp and Mudge (1921) as a useful differential test, and this method was fruitfully applied by these investigators in their subsequent studies. As ammonia production from peptone has been used by only a few investigators in recent years, it cannot be said just how much general utility it has in the study of the streptococci. However, it appears to be distinctly valuable in connection with the "viridans" types, the better known members of which do not produce ammonia, and it also has a value in the study of the lactic-acid streptococci.

The uses of the temperature limits of growth and methylene-blue tolerance of streptococci were introduced by Sherman and Albus (1918). Tolerance to sodium chloride and the alkaline pH limits of growth (Sherman, 1921) have been used by a few workers for years in the differentiation of *Lactobacillus bulgaricus* from certain closely related lactobacilli, but these tests have only recently been systematically applied, at different levels, to the study of streptococci. (Sherman and Stark, 1934; Sherman, Mauer and Stark, 1937; Sherman, Stark and Mauer, 1937; Yawger and Sherman, 1937b.) The methylene-blue milk test has been used in a number of ways. The work of Ayers and his associates showed that while the test as applied by

Sherman and Albus worked well in differentiating such sensitive types as *Streptococcus pyogenes* and *Streptococcus mastitidis*, on the one hand, from such tolerant forms as *Streptococcus lactis* and *Streptococcus fecalis* on the other, it had no special value when applied to miscellaneous non-hemolytic streptococci such as *Streptococcus bovis*. Avery (1929a) tried methylene blue in three different concentrations and concluded that a 1:5,000 solution in skimmed milk has a value in differentiating certain tolerant hemolytic streptococci found in dairy products from the human and bovine pathogenic types, which were inhibited. Avery (1929b) did not find the test as used by him of value when applied to the non-hemolytic streptococci. Edwards (1933) has used, with fruitful results, a dilute solution of methylene blue in a broth medium for differentiating the hemolytic streptococci belonging to the Lancefield group C. Recently it has been shown that if the concentration of methylene blue in skimmed milk is increased to 0.1 per cent, the test has some value in its application to the streptococci as a whole; all known streptococci appear to be inhibited except the members of the lactic and enterococcus groups. (Sherman, Mauer and Stark, 1937; Sherman, Stark and Mauer, 1937; Sherman and Wing, 1937; Yawger and Sherman, 1937b.)

Some streptococci have much more tolerance for bile than do others, and bile media have been fairly extensively used in the differentiation of these organisms. The enterococci show outstanding tolerance to bile, but this characteristic appears to be shared, at least to a considerable degree, by a number of other streptococci such as *Streptococcus bovis*, *Streptococcus lactis* and *Streptococcus mastitidis*. The origin of the use of bile in the study of streptococci is not clear. More than twenty-five years ago, bile was used as a selective medium for the detection of fecal streptococci in milk (Kinyoun and Dieter, 1912). A little later it was observed that the mastitis streptococcus also had considerable tolerance for bile and that the test had some value for the detection of this organism in milk coming from infected udders. (Rogers, Clark and Evans, 1916.) Because of the known tolerance of the mastitis streptococcus to bile, Sherman

and Albus (1918) used a bile medium in an attempt to differentiate this organism from the lactic-acid streptococcus, but found that *Streptococcus lactis* was also tolerant to bile, in fact even more so than the udder streptococci under the conditions employed by them. These facts have been rediscovered and put to use more systematically in the study of streptococci in recent years. Weissenbach (1918) observed the tolerance of the enterococci to bile and recommended a bile medium for the differentiation of these streptococci; Meyer and Schönfeld (1926) again noted the bile tolerance of *Streptococcus lactis*; and only recently the greater bile tolerance of *Streptococcus mastitidis*, as compared with some of the other closely related hemolytic streptococci, has again come to light. Recent investigators of the hemolytic streptococci have employed with some success the bile-blood-agar medium of Belenky and Popowa (1929). The hemolytic enterococci and *Streptococcus mastitidis* grow on this medium whereas the representatives of the other hemolytic species usually do not; whether or not this medium has much value in its application to the streptococci as a whole has not been determined. Houston (1934) states that the ability to grow in pure bile with the production of long chains is a valuable characteristic for the identification of enterococci. This suggestion is worthy of systematic investigation, but it appears from Houston's paper that some of his organisms (the "Bergen streptococcus") were probably related to *Streptococcus bovis* rather than being true enterococci; and it should be recalled that bile media were long ago shown to induce chain formation in *Streptococcus lactis* (Sherman and Albus, 1918). Although of limited usefulness, bile tolerance tests have proved valuable for certain specific purposes in the study of streptococci and it is probable that systematic investigations would reveal further special applications. Safford (1937), for example, has obtained preliminary results which indicate that the greater bile tolerance of *Streptococcus bovis* may be of value as another means of differentiating this organism from *Streptococcus salivarius*.

The ability of streptococci to split esculin has been curiously mixed with bile tolerance. Stemming back to the use of an escu-

lin-bile-salts medium for colon bacteria by Harrison and Vanderleck (1909), many workers have studied the ability of streptococci to hydrolyze esculin in media containing bile salts. Obviously, the ability to attack esculin is one thing and the ability to grow in the presence of bile is quite another. This fact has doubtless been recognized by many workers and some of them (Weatherall and Dible, 1929; S. J. Edwards, 1932) have specifically called attention to the fact that the method as generally used is a test for bile tolerance rather than for the ability to attack esculin. The data given on esculin in this review are taken from those investigators who have applied it in the absence of bile in order to determine whether or not the streptococci could actually attack it. As esculin is very generally attacked by streptococci under favorable conditions, it does not appear to have much differential value, but the apparently consistent inability of a few types such as *Streptococcus mastitidis* and *Streptococcus thermophilus* to attack it gives esculin a limited value.

The use of starch as a fermentation test, that is, a test for the production of acid from starch, has long been used by some workers with the streptococci, but has generally been recognized as an unsatisfactory method when employed in this way. Another old method in the study of bacteria is a test for their ability to hydrolyze starch in an agar medium. It is probable that this test was applied to the streptococci by earlier workers, but its systematic application is of more recent origin. Andrewes (1930) found only 20 of 140 hemolytic streptococci from human infections able to hydrolyze starch; and only a few strains of the non-hemolytic streptococci tested had this ability. This method was also revived by Sherman and Stark (1931) who found it to have especial value in the identification of *Streptococcus bovis*, which actively hydrolyzes starch; and the test has been widely applied by these workers and their associates in the study of streptococci, especially the non-hemolytic types. As the enterococci and the lactic-acid streptococci do not hydrolyze starch, and this property is found in only certain members of the viridans group, the ability to hydrolyze starch has considerable value in the study of non-hemolytic streptococci.

Its value in connection with the hemolytic streptococci is not clear, but from the limited information at hand it appears that starch is more frequently attacked by the hemolytic types. At any rate, the starch-agar test presents a method which deserves to be carefully investigated.

The discovery of the lytic action on human fibrin by *Streptococcus pyogenes* and certain other types (Tillett and Garner, 1933), together with the further clarification of this phenomenon (Garner and Tillett, 1934a, 1934b), constitutes a brilliant contribution to the knowledge of streptococci and furnishes a valuable addition to the methods of studying these organisms. Some cultures of *Streptococcus pyogenes* are not strongly fibrinolytic, but such strains are relatively rare; it is also true that occasional strains of various species of hemolytic streptococci may show slow fibrinolysis when the tubes are incubated at 37°C. for 24 hours; it is further true that if the technique is modified in certain ways (Neter and Witebsky, 1936) various streptococci may show some action on fibrin; but if the technique of Tillett and Garner is adhered to, and arbitrary distinction is drawn between rapid fibrinolysis and weak or slow digestion, the only hemolytic streptococci which are known to be actively fibrinolytic are *Streptococcus pyogenes* (Lancefield group A) and certain types belonging to the Lancefield groups C and G. This valuable test perhaps needs further standardization in order to bring out to the fullest extent its differential potentialities, but as it now stands it is not only an extremely useful reaction in the classification of the streptococci, but valuable as a presumptive test for those which are of most importance as agents of human disease. The conclusion of Davis and Guzdar (1936) that this test did not have much value in differentiating the streptococci which are most important as human pathogens (Lancefield group A) appears to have been drawn from a faulty perspective. It so happened that the collection of hemolytic streptococci with which these investigators worked was made up entirely of representatives of the Lancefield groups A, C and G—their organisms, in addition to group A, representing the only other groups which are known to contain actively fibrinoly-

tic members. In the tables contained in this paper, positive fibrinolysis is limited, arbitrarily, to those organisms which cause an active and rapid lysis.

The most important contribution to methods for the classification of streptococci is the serological technique of Lancefield (1933) which divides the hemolytic streptococci into groups, by means of a precipitin reaction, based on the presence in these organisms of a group-specific polysaccharide—the “C substance”—which cuts lines, though more perfectly, very closely simulating those which have long been drawn between species of streptococci. The Lancefield method in no sense upsets what was previously known, but puts it on a much firmer basis and extends it, at the same time brilliantly pointing out the location of new groups or species of which bacteriologists were previously quite unaware. The soluble specific substance, upon which the grouping of the hemolytic streptococci is based, was first found by Hitchcock (1924a) as a “residue antigen” and was believed by him to be common to practically all hemolytic streptococci. This view was held until its group-specific nature was discovered by Lancefield (1933). Although the grouping of the hemolytic streptococci by means of the species-specific antigen may be considered, from the standpoint of the taxonomist, as her crowning achievement, this accomplishment of Lancefield developed from a series of penetrating studies on the antigenic structure of non-hemolytic and hemolytic streptococci (1925a, b, 1928a, b, c, d, e). With these other equally important phases of the immunological aspects of the streptococci we are not specifically concerned for the purposes of this review. The importance of the organisms dealt with, the complexity of the problem, and the scientific and practical value of the results achieved, rank the singlehanded Lancefield work not only alone among contributions to the taxonomy of streptococci, but with few peers in the annals of modern bacteriology.

It is too early to accept the Lancefield method as one giving infallible decisions, but there can be little doubt that it attains an accuracy in the classification of the hemolytic streptococci which is far beyond that heretofore possible. Whether an occa-

sional strain is so lacking in group antigen as to defy identification, or whether the method may fail on rare occasions for some other reason, will be revealed as experience with the technique accumulates. Although Reich (1935), in a preliminary paper, reported what he considered to be temporary changes in the Lancefield grouping of a hemolytic streptococcus, by rabbit passage, this has not been verified by full publication; whereas Gay and Clark (1937) have reported the failure to obtain such changes by passage through rabbits over a prolonged period. As yet the method has not been seriously challenged; and in the meantime it has proved its worth not only in bacterial taxonomy but in the practical solution of epidemiological problems (Swift, Lancefield and Goodner 1935).

Bacteriophages have been used successfully by several investigators for certain differentiations among the streptococci and this method is now being systematically explored by Evans (Hadley and Dabney, 1926; Clark and Clark, 1927; Shwartzman, 1927; Lancefield, 1932, 1933; Evans, 1934, 1936a). Just how much value this method has in its application to the streptococci as a whole will doubtless become clear in the near future.

A method which probably would have value in the systematic study of streptococci is a simple and reliable test for the possession of flagella—rather than motility, since some flagellated bacteria are not constantly motile. Since the first claims of motility in streptococci by Ellis (1902a,b) there have been intermittent reports of this by other workers. That motility exists in some streptococci has been proved by a number of workers; whether such motile strains represent separate species, or whether some of the ordinary streptococci are motile under certain conditions, is not yet fully clear. Most of the motile streptococci have been isolated from milk, cheese or feces, and have been related to the lactic-acid streptococci (Flatzek, 1919; Schiebllich, 1932) or the enterococci (Koblmüller, 1935; Levenson, 1937). Stölting (1935), however, considered his strains to be atypical forms of *Streptococcus bovis*, and found them to be different from the motile streptococcus of Schiebllich (1932). A motile streptococcus isolated from milk by Dr. C. B. Van Niel, which

has been studied in this laboratory, was found to agree in all essential physiological characteristics with *Streptococcus fecalis* and to fall in the same serological group by the Lancefield method as our stock cultures of this species.

#### PRIMARY DIVISIONS OF THE STREPTOCOCCI

A major factor which has rendered abortive attempts to classify the streptococci as a whole has been the failure to segregate, first, in more or less homogeneous divisions, the members of the genus. This is especially the case in those classifications in which the fermentation tests have constituted the major criteria on which the identifications were based. Though valuable in their proper rôle of aiding in the differentiation of closely related types, the fermentation tests when applied to the streptococci as a whole, without previous subdivision by other methods, can lead only to utter confusion. So far as the non-hemolytic streptococci are concerned, some such subdivision as the one here suggested is absolutely essential if any order is to be obtained in their classification; and though not so essential in the case of the hemolytic streptococci, such an approach is valuable in order to segregate at once the hemolytic members of the enterococcus division from those distantly related hemolytic groups which may for convenience be termed the "pyogenic streptococci."

It should be emphasized that there is nothing "official" about this suggested approach to the classification of the streptococci. It has been in use for the past decade in the reviewer's laboratory, but it has not been used by others until very recently, and hence no claims can be made that such a division of the streptococci represents any united opinion of bacteriologists. However, such a primary division does serve the important purpose of getting the related streptococci together in manageable groups, and does not in any way conflict with other methods of classification. These divisions were originally based on the temperature limits of growth, supplemented by other basic characteristics; and the temperature requirements, though not



infallible, have proved to be among the most important properties of the streptococci for differential purposes (Sherman, 1937).

TABLE 1  
Divisions of the streptococci

DIVISION	GROUP OR SPECIES	LANCFIELD GROUP	HEMOLYSIS	GROWTH AT		GROWTH IN PRESENCE OF			STRONG REDUCTION	SURVIVAL 60°C., 30 MINUTES	NH <sub>4</sub> FROM PEPTONE
				10°C.	45°C.	6.5 per cent NaCl	pH 9.6	0.1 per cent methylene blue			
Pyogenic	<i>S. pyogenes</i>	A	+	-	-	-	-	-	-	-	+
	<i>S. mastitidis</i>	B	±	-	-	-	-	-	-	-	+
	<i>S. equi</i>	C	+	-	-	-	-	-	-	-	-
	"Animal pyogenes"	C	+	-	-	-	-	-	-	-	+
	The "human C"	C	+	-	-	-	-	-	-	-	+
	"Minute hemolytic"	F	+	-	*	-	-	-	-	-	+
	Group G streptococci	G	+	-	*	-	-	-	-	-	+
	Group E streptococci	E	+	-	-	-	-	-	-	-	+
	Group H streptococci†	H	±	-	+	-	-	-	-	±	±
Viridans	<i>S. salivarius</i>		-	-	±	-	-	-	-	-	-
	<i>S. equinus</i>		-	-	+	-	-	-	-	±	-
	<i>S. bovis</i>		-	-	+	-	-	-	-	+	-
	Varieties of <i>S. bovis</i>		-	-	+	-	-	-	-	+	-
	<i>S. thermophilus</i>		-	-	+	-	-	-	-	+	-
Lactic	<i>S. lactis</i>		-	+	-	-	-	+	+	+	+
	<i>S. cremoris</i>		-	+	-	-	-	+	+	±	-
Enterococcus	<i>S. fecalis</i>		-	+	+	+	+	+	+	+	+
	<i>S. liquefaciens</i>		-	+	+	+	+	+	+	+	+
	<i>S. zymogenes</i>	D	+	+	+	+	+	+	+	+	+
	<i>S. durans</i>		+	+	+	+	+	+	-	+	+

\* Indicates occasional variation from type reaction. Extremely rare exceptions (e.g., *Bacterium coli*, lactose -) not noted.

† Group H streptococci, of doubtful status with respect to hemolysis, also fall between the "pyogenic" and "viridans" streptococci in physiological characteristics.

In table 1 are given the types or species of streptococci grouped in their respective divisions. The characteristics used in this table are those which are of primary value in separating

the divisions from one another; the further differentiation of the individual species is developed in later tables.

It would seem from an inspection of table 1 that the suggested separation of the streptococci into major divisions has a logical basis. Of these, the enterococci certainly represent a clearly defined division of the streptococci; the lactic streptococci represent another group which is nearly if not quite so clearly defined; less can be said for the so-called "pyogenic" and the "viridans" divisions, but they also appear to represent more or less "natural" subdivisions of the streptococci.

The enterococci are markedly differentiated from the other known species of the streptococci by their combination of low minimum and high maximum temperatures of growth and by their greater tolerance to salt and alkali. From most streptococci, they also differ in having high thermal death points, in being resistant to relatively concentrated solutions of methylene blue, and in usually having strong reducing action.

The lactic streptococci have in common with the enterococci low minimum temperatures of growth, strong reducing action, and methylene-blue tolerance; but the lactic streptococci differ in having low maximum temperatures of growth and less tolerance to salt and alkali. As will be shown later, the lactic and enterococcus streptococci are also less perfectly separated by certain fermentation tests and other characteristics.

What are termed the "pyogenic" streptococci at least represent a division which conforms with past usage, as it has long been the custom to consider these species closely related. They are usually hemolytic; do not grow at 10°C., and usually do not grow at 45°C.; have low thermal death points; have weak reducing action; and are not tolerant to methylene blue, salt, or alkali.

The "viridans" streptococci differ from the pyogenic types in not being beta hemolytic, in usually growing at 45°C., and in being unable to produce ammonia from peptone. Some members of this division also have high thermal death points. Like the pyogenic species, the "viridans" streptococci cannot grow at 10°C., have weak reducing power, and are not tolerant to methylene blue, salt, or alkali.

In considering the natural divisions of the streptococci it is of some pertinence to consider the special status of the enterococci. The term *Enterococcus* is frequently used as a separate generic designation quite outside of the genus *Streptococcus*. Such names as "*Enterococcus fecalis*" and "*Enterococcus hemolyticus*" have crept into the literature. Without question, the enterococci represent a clearly defined division of the streptococci, probably the most clearly marked subdivision of the whole genus. But when it comes to considering the enterococci as an independent genus one may well question where, precisely, the line is to be drawn. With the exception of certain tests which have only recently been systematically used in the differentiation of the enterococci, none of the properties which have been considered especially characteristic of the enterococci are peculiar to them: Most certainly, the enterococci could not be established as a separate genus on the basis of morphological and general cultural characteristics; low minimum temperatures of growth, strong reducing action, and methylene-blue tolerance are shared by the lactic streptococci; high thermal death points and high maximum temperatures of growth are also characteristic of *Streptococcus bovis* and *Streptococcus thermophilus*, which are more closely related to *Streptococcus salivarius* and only rather remotely related physiologically to the enterococci; bile tolerance, at best of little value in the differentiation of streptococci, is not limited to the enterococci, as *Streptococcus mastitidis*, *Streptococcus bovis* and *Streptococcus lactis* are also tolerant to the concentrations of bile which are commonly used in the study of streptococci; and most positively a generic differentiation could not be based on habitat as many streptococci occur in the intestines and such species as *Streptococcus bovis* and *Streptococcus equinus* are just as truly intestinal forms as are the enterococci. For the present at least, it would be hard to justify a generic segregation of the enterococci from the other streptococci.

#### THE PYOGENIC STREPTOCOCCI

Putting together in one division all of the hemolytic streptococci with the exception of the hemolytic enterococci is in accord-

ance with custom. In fact, the majority of those who have dealt with what they have termed "*Streptococcus hemolyticus*" have not even excluded the hemolytic enterococci from their supposedly homogeneous group. The term, "pyogenic streptococci," as here used, at least limits the division to somewhat related organisms, though it must be admitted that the relationship between some of the members of this division probably is not very close.

The attempted division of these streptococci into "species" is based primarily on the Lancefield serological grouping supplemented by cultural and biochemical tests. There is no conflict between the serological and the physiological approaches; rather they are complementary. In some of the Lancefield groups, such as A, B and F, the serological grouping and the physiological tests agree well in defining rather homogeneous species or groups. On the other hand, in such groups as C and G the physiological tests are of value for the subdivision of the serological groups; most importantly, the Lancefield technique enables the cutting of certain vital lines, such as those between members of group A (*Streptococcus pyogenes*) and certain strains belonging to groups C and G, for which purpose the physiological tests thus far at hand have been unable adequately to function.

Since the "species" grouping of these streptococci is based primarily on the Lancefield grouping, the review of this division must of necessity deal principally with recent work.

In table 2 are presented data on the characteristics of members of the several species or groups belonging to this division.

Because in recent years the hemolytic streptococci have been studied with fewer physiological tests than have the non-hemolytic forms, the assembling of adequate data on the biochemical characteristics of the pyogenic streptococci is difficult, and the data presented are woefully inadequate. Much of the material presented in this table has been obtained in the reviewer's laboratory through the study of comparatively few representative cultures belonging to the various groups. The compilation undoubtedly contains a number of errors as well as being incomplete; it is, however, presented without apology for what

it may be worth, and it may perhaps prove of some value in indicating a few points on which it would be desirable to extend our knowledge. For example, it is possible that an extension of the test substances ordinarily used to include such compounds as glycerol, raffinose and starch might aid in lessening the confusion between *Streptococcus pyogenes* and those members of the Lancefield groups C and G from which its differentiation is not clear on the basis of the conventional tests now in vogue.

TABLE 2  
*The pyogenic streptococci: additional characteristics*

GROUP OR SPECIES	LANCIEFIELD GROUP	ACTIVE FIBRINOLYSIS	SODIUM HYDROLYSATE HYDROLYZED	STARCH HYDROLYZED	ESCULIN SPLIT	GROWTH ON 40 PER CENT BILE-BLOOD AGAR	GELATIN LIQUEFIED	MILK CURDLED	FINAL pH IN GLUCOSE BROTH	ACID PRODUCED FROM									
										Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol
<i>S. pyogenes</i> .....	A	+	-	?	±	-	-	-	6.0-4.8	-	+	+	+	+	-	-	?	±	±
<i>S. mastitidis</i> .....	B	-	+	-	-	+	+	+	4.8-4.2	-	+	+	+	+	-	-	-	-	±
<i>S. equi</i> .....	C	-	-	-	-	-	-	-	5.5-4.8	-	+	+	+	+	-	-	-	-	±
"Animal pyogenes"....	C	-	-	+	+	-	-	-	5.0-4.6	-	±	+	+	-	-	-	-	+	+
The "human C".....	C	±	-	±	±	±	-	±	5.4-4.6	-	+	+	±	-	-	-	+	-	±
"Minute hemolytic"....	F	-	-	+	+	+	-	-	5.4-4.6	-	+	+	±	±	-	-	-	-	±
Group G streptococci.	G	±	-	±	±	±	-	-	6.0-4.6	-	+	+	±	+	±	-	±	-	+
Group E streptococci.	E	-	-	±	±	-	-	-	4.8-4.2	-	±	+	+	+	-	-	±	-	±
Group H streptococci.	H	-	-	-	±	-	-	-	5.0-4.5	-	+	+	±	±	+	-	-	±	+

\* See table 1.

### *Streptococcus pyogenes* (Lancefield Group A)

There is still a difference of opinion as to whether the most important human pathogenic streptococci, all members of the Lancefield group A, should be considered as constituting one or more than one species. Without prejudice, and in accordance with what appears to be the prevailing opinion at the present time, they will be considered as belonging to one species, *Streptococcus pyogenes*.

*Streptococcus pyogenes* (Rosenbach) has long been known "in location," so to speak; when isolated from active and severe human infections, there is little doubt that the organism which carries this name has been identified with a high percentage of

accuracy during the years. Within the past fifteen years this species has become more clearly defined on the basis of its physiological characteristics but, as recent investigations have shown, in a small proportion of cases the identification can be made unequivocal only by the application of the Lancefield technique.

So far as is known, *Streptococcus pyogenes* is strictly a human pathogen under natural conditions, though spontaneous infections in laboratory animals have been noted. However, as was shown experimentally by Davis and Capps (1914), it is well known that the udders of cattle may become infected from human attendants, thus in turn serving as an animal source of infection with this organism among people, through the consumption of infected milk. Even in this case, the infection does not appear to be self-sustaining by passing from animal to animal so as to maintain a more or less permanent source of infection. Edwards (1935) studied three cultures, from baby chicks with bronchitis, which belonged to the Lancefield group A. These cultures differed from the typical form of *Streptococcus pyogenes* in that they did not ferment lactose, and only one of them was actively fibrinolytic.

As previously indicated, with the exception of the Lancefield serological grouping, *Streptococcus pyogenes* cannot as yet be identified with absolute certainty by means of simple physiological tests. However, if this organism can be identified on the basis of what it is (as shown by serological methods) it should certainly be possible to find a way of identifying it on the basis of what it does. In a sense, such distinguishing functional characteristics are now known for *Streptococcus pyogenes*: its specific and unique disease relationship in man is an expression of physiological attributes not possessed by other streptococci; also, as Todd (1934) has shown and Long and Bliss (1937a) have verified, *Streptococcus pyogenes* (Lancefield Group A) is the only streptococcus among the various hemolytic groups which is known to produce an antigenic streptolysin.

Although *Streptococcus pyogenes* may be readily confused with a number of other hemolytic streptococci when only the conventional physiological tests are used, this confusion can be very

largely, but not entirely, eliminated by the use of a greater variety of the imperfect tests now at hand. For example, attention to such characteristics as the temperature limits of growth, action on milk, and the fermentation of raffinose, would eliminate a considerable amount of this confusion. Of great value in this connection is the fibrinolytic test of Tillett and Garner. Although occasional strains of *Streptococcus pyogenes* are encountered which do not have this property, the ability to lyse human fibrin is an outstanding characteristic of this organism; and if the interpretation of a positive test is limited to those streptococci which are very actively fibrinolytic, the only other streptococci known to have this action are certain types found in the Lancefield groups C and G. Obviously, some additional tests to supplement fibrinolytic action are most desirable at this point. The use of methylene blue as a tolerance test in a somewhat more dilute solution than that now in most general use (1:5,000) would be of some help, but would fall far short of satisfying the need. Since many of the fibrinolytic members of groups C and G ferment glycerol, and some also hydrolyze starch, it would be of some value to have more explicit information on the action of *Streptococcus pyogenes* on these substances. Although frequent references are made in the older literature to the fermentation of glycerol by some strains of *Streptococcus pyogenes*, information on this point since the Lancefield technique has been available is almost completely lacking. With respect to the hydrolysis of starch, Andrewes (1930) found only 20 of 140 hemolytic streptococci from human sources to have this action; but one cannot be sure that these starch-hydrolyzing strains were actually members of the Lancefield group A, though some of them had come from cases of scarlet fever, puerperal sepsis and erysipelas, and three of Griffith's types of scarlet fever streptococci were represented.

*Streptococcus pyogenes*, signifying by this term all of the members of the Lancefield group A, represents a group of streptococci showing rather diverse reactions in the fermentation tests. Strains which fail to ferment lactose or salicin and others which do ferment mannitol have long been recognized, and these vari-

ations have been confirmed since the advent of the Lancefield method of grouping. The trehalose and sorbitol tests have also been violated by a few authentic strains of group A streptococci (Lancefield and Hare, 1935; Davis and Guzdar, 1936). Although the present consensus of opinion is rather emphatically in favor of considering all members of group A as belonging to the one species, *Streptococcus pyogenes*, a few years ago there was a rather strong tendency toward a division of this group into several species. Among the factors giving impetus to this movement may be mentioned the work of Davis (1912 and subsequent papers) on epidemic sore throat, that of Dick and Dick (1924) on scarlet fever, and the investigations of Birkhaug (1925a, b, c, 1926) on erysipelas. It would seem that the evidence at this point must have been rather convincing in order to force such a sound and conservative investigator as Hektoen (1930) to the definite conclusion that "... etiologically erysipelas and scarlet fever are as distinct and different as they are clinically."

Among the different "species" which were set up within the "pyogenes group," the one which perhaps had the best claim for recognition on tangible morphological and cultural grounds was the *Streptococcus epidemicus* of Davis. This organism was supposed to be especially characterized by the formation of capsules and the production of moist mucoid colonies. However, it gradually became recognized that the special characteristics of this organism were properties which came within the bounds of natural variation in the streptococci; and through the works of various investigators, among which may be mentioned particularly that of Williams and Gurley (1932), *Streptococcus epidemicus* lost standing as an independent type.

However, it is not yet safe to draw definite conclusions concerning the advisability of recognizing more than one species type in the "pyogenes" or Lancefield A group. Evans (1937) has just put forward the claims of *Streptococcus scarlatinae* for recognition as a separate species, and the implication is given in a previous paper (1936b) that the claims of other types for species recognition will be presented in forthcoming communica-



tions. As defined by Evans, *Streptococcus scarlatinae* is differentiated from *Streptococcus pyogenes* by its inability to ferment salicin. Although she based the differentiation of *Streptococcus scarlatinae* on the salicin fermentation test, Dr. Evans cited certain other average differences between *Streptococcus scarlatinae* and *Streptococcus pyogenes* which tend to support the separation made: The cultures designated as *Streptococcus scarlatinae* were found to have on the average less virulence, weaker fibrinolytic action, and more tolerance to bile than did the cultures which were considered as *Streptococcus pyogenes*. Though not the exclusive cause of scarlet fever, the salicin-negative strains are claimed to occur rarely in diseases other than scarlet fever or sore throat without rash.

It should be noted that according to the Holman (1916) classification a salicin-non-fermenting organism which is otherwise identical with *Streptococcus pyogenes* would be designated as *Streptococcus anginosus*. With some reason, it would appear, Evans rejects the name of *Streptococcus anginosus* in favor of *Streptococcus scarlatinae* which she believes should have priority for an organism of this type. As will be noted later, there is also some reason to believe that the original *Streptococcus anginosus* of Andrewes and Horder (1906) was an organism which belonged quite outside of the Lancefield group A.

In connection with the propriety of establishing separate species within the Lancefield group A, it is of some pertinence to consider the matter of the serological types within this group, which all agree represent only intra-species varieties. Dochez, Avery and Lancefield (1919) separated four biological types of *Streptococcus pyogenes* by means of agglutination and mouse-protection tests. Lancefield (1928a) also divided this group into types by means of the precipitin test based upon a type-specific protein antigen (the "M substance") which she discovered. The types established by the precipitin reaction were correlated by Lancefield with the biological types mentioned above. By means of his slide agglutination technique Griffith (1934) established 27 serological types of *Streptococcus pyogenes*, and stated that probably more than 30 of such types exist. Al-

though it appears from later developments that a few of Griffith's types do not belong to the Lancefield group A (Hare, 1935), Pauli and Coburn (1937) have established 28 serological types of authentic group A streptococci. It has been clearly shown that the type-specific antigen may be lost in whole or in part, and within limits regained, by cultures under artificial cultivation. (Lancefield, 1928a; Lancefield and Todd, 1928; Todd and Lancefield, 1928.) It would seem that the loss or acquisition of a specific protein by the cellular complex of a bacterium represents as great a degree of variation as does the loss or suppression of the ability to ferment a particular carbohydrate.

As habitat has long been considered germane to the subject of taxonomy, it is appropriate as well as of interest to review what has been learned about the natural distribution of *Streptococcus pyogenes* since its identification has been made more positive by the Lancefield grouping method. The information thus far at hand indicates that, aside from active infections, the human throat is probably the principal reservoir for the maintenance of this organism. Hare (1935) found that about one-third of the hemolytic streptococci isolated from the normal human nose and throat belonged to the Lancefield group A. Davis and Guzdar (1936) found group A hemolytic streptococci in about three per cent of the throats of 788 apparently healthy Chinese. Foote, Welch, West and Borman (1936) found that more than 20 per cent of 85 milk handlers harbored group A hemolytic streptococci in the nose or throat at least once during three months of weekly tests; five of the 20 individuals who gave positive tests for group A streptococci appeared to be constant carriers of these organisms. Lancefield and Hare (1935) rarely recovered group A streptococci from the vagina, except in cases of active infection; Hare and Maxted (1935) likewise did not find *Streptococcus pyogenes* in the feces of normal people, though it is frequently isolated from the feces in cases of throat infections such as scarlet fever; and Colebrook, Maxted and Johns (1935) failed to isolate the group A streptococcus from the perianal and perineal skin of 160 women, thus again indicating the general absence of this organism in the intestine and the birth

canal. The latter investigators did, however, obtain *Streptococcus pyogenes* from the hands of seven of 181 normal individuals, but it was believed probable that the organism in these cases was derived from the respiratory tract.

*Streptococcus agalactiae* or *Streptococcus mastitidis* (Lancefield Group B)

In years past, *Streptococcus mastitidis* was the name most commonly applied to the organism which is usually associated with bovine mastitis, but the tendency in recent years, both in Europe and America, has been towards the use of *Streptococcus agalactiae*. It would appear from Hansen's (1935) summary of the nomenclature of this organism that though the former name has some claims for priority on the basis of its use in trinomial form, *Streptococcus agalactiae* was earlier reduced to a binomial. As Hansen points out, however, both of these might be considered invalid on the basis of priority because of the generally unknown name, *Streptococcus nocardii*, which Trevisan (1889) had previously given to this organism. As it can of course be reasonably argued that none of the early descriptions was complete in a modern sense, the final agreement, if any, as to the proper name will doubtless be arrived at through custom and usage.

For our present purposes, it is quite impossible to review comprehensively the work on the mastitis streptococcus; but we are not primarily concerned with the historical aspects of the subject, and reference may be made to such reviews as those by Seelemann (1932), Hansen (1935), and others. The useful bibliography compiled by Hansen (1934) contains references to more than one thousand papers which deal in one way or another with the subject of mastitis. We shall, therefore, consider very briefly the taxonomic aspects of the organism, with especial attention to the newer facts which have been learned about it since the Lancefield method of serological grouping has been available.

Although some good descriptions of the organism may be found in earlier works, the precise identity of *Streptococcus*

*mastitidis* may well be dated from the work of Ayers and his co-workers, who introduced new tests and clearly differentiated this organism from the *Streptococcus pyogenes* of human infections. (Ayers, Johnson and Davis, 1918; Ayers and Rupp, 1922; Ayers and Mudge, 1922.) These investigators correlated the production of a low pH in glucose broth, limited and variable hemolytic power, and the ability to hydrolyze sodium hippurate, in the differentiation of the mastitis streptococcus from hemolytic streptococci from human infections. The ability to produce a low final pH was quickly confirmed by others (Avery and Cullen, 1919; Brown, 1920) and the sodium hippurate reaction has remained, for its specific purpose, one of the outstanding differential tests used in bacteriology.

The diverse action on blood by various strains of *Streptococcus mastitidis* has been supported by the work of many investigators since Ayers and his associates, and that such variation occurs within the species has also been confirmed serologically. Stableforth (1932) showed that hemolytic and non-hemolytic strains frequently were of the same serological type; and Lancefield (1934b) has reported the loss of the property of hemolysin production in a culture of *Streptococcus mastitidis* without change in immunological specificity as to group and type.

The production of pigment, ranging in color from yellow to brick-red, is a rather characteristic property of *Streptococcus agalactiae*, and references to this are found in some of the early descriptions of this organism. Among relatively recent investigators, Orla-Jensen (1919) mentioned the production of an orange color in casein-peptone broth containing soluble starch, as being very characteristic of his cultures of *Streptococcus mastitidis*. However, all strains are not chromogenic, and Lancefield (1934b) has shown further that the property of producing pigment may be lost in laboratory cultures. Another characteristic which appears to make the mastitis organism somewhat unique among the hemolytic streptococci is its apparent total inability to attack esculin. (Diernhofer, 1932; Orla-Jensen, 1934; Hansen, 1935.)

Aside from its occurrence in bovine udders showing definite

signs of infection, the mastitis streptococcus has long been known to occur in the udders of a large proportion of cattle which appear, superficially at least, to be entirely normal (Sherman and Hastings, 1915; Evans, 1916). These observations have been confirmed by many workers, though it is a nice question just what may be considered a "normal" animal. However, Hucker (1937a) has reported the isolation of *Streptococcus agalactiae* from the aseptically-removed udder tissues of cows known to be free of mastitis. In some cases the organism was also obtained from the udder tissues of virgin heifers. The mastitis streptococcus is not known to occur in the bovine mouth or intestine, but in view of the information which has recently come to light concerning the harboring of this organism by human beings, the possibility should be recognized that there may be other animal reservoirs for this organism, aside from the udder.

Lancefield group B streptococci, physiologically and serologically entirely typical of *Streptococcus mastitidis* or *Streptococcus agalactiae*, have been isolated from a number of human sources: from the nose and throat (Lancefield, 1933; Plummer, 1935; Hare, 1935); from the vagina (Lancefield and Hare, 1935); and from feces (Smith and Sherman, 1937). Although *Streptococcus agalactiae* most certainly cannot be looked upon as a human pathogen, as in the case of a number of other "non-pathogenic" streptococci, the possibility of its rare occurrence in human infections should be recognized. Lancefield and Hare (1935) noted a few cases in which it seemed possible that group B streptococci were the cause of mild infections of the uterus, and Hare (1935) has since reported two cases of fatal uterine infection following childbirth.

Stableforth (1932) and Lancefield (1934a) studied the serological types of *Streptococcus agalactiae* by means of agglutination and precipitin tests, each establishing three types within the group (the type-specific antigen in group B—the "S substance"—being a polysaccharide, Lancefield, 1934a). Stableforth (1937) again investigated this problem with a larger assortment of cultures and obtained a total of five types consisting of three main types, two of which contained related subtypes. Stewart

(1937) studied 72 strains from Australia and found four types represented.

In spite of some variability with certain tests, such as its reactions on blood and salicin, *Streptococcus agalactiae* appears to represent a clearly defined and homogeneous group of organisms. Nevertheless, there can be little doubt that a number of the "species" of hemolytic streptococci which have been reported from the bovine udder have in fact been strains of *Streptococcus agalactiae* which varied slightly from type in some of the biochemical tests. This is true of at least some of the strains which have been described under the name of *Streptococcus asalignus* (Frost, Gumm and Thomas, 1927). Two cultures of *Streptococcus asalignus*, kindly furnished by Dr. Frost, were found to belong to the Lancefield group B, when tested serologically in our laboratory, and to be typical physiologically of *Streptococcus mastitidis* or *Streptococcus agalactiae*, though they were more strongly hemolytic than is usual for members of this species, and also failed to ferment salicin.

Plastringe and Hartsell (1937) have very recently described as a new species, *Streptococcus pseudo-agalactiae*, an organism obtained from the bovine udder. In biochemical reactions this organism is the same as *Streptococcus agalactiae* except that milk is usually not curdled, though sometimes coagulation takes place after incubation for seven days; also, there was frequently a slight reduction of methylene blue which was not caused by typical cultures of *Streptococcus agalactiae*. Plastringe and Hartsell state: "Although the *S. pseudo-agalactiae* cultures were usually somewhat less hemolytic than the *S. agalactiae* cultures, and the reactions of the two organisms in litmus milk and methylene blue milk were at times slightly different, none of the biochemical tests gave results which were sufficiently clear-cut for differential purposes."

Serologically, *Streptococcus pseudo-agalactiae* is believed by Plastringe and Hartsell to represent a distinct group. By the Lancefield method, extracts of *Streptococcus pseudo-agalactiae* gave slight or partial reactions with both groups B and C antisera, good reactions, however, being obtained with antisera pre-

pared against cultures of *Streptococcus pseudo-agalactiae*. On the other hand, extracts of typical group B organisms gave only partial or negative reactions when tested with antisera prepared against *Streptococcus pseudo-agalactiae*. Another reason given by Plastringe and Hartsell for considering their organism a new species is that it appears to be less frequently associated with mastitis than does the typical *Streptococcus agalactiae*; some of the udders yielding the pseudo-agalactiae type show no laboratory evidence of mastitis, and the infection, with or without evidence of mastitis, is usually of short duration.

It is too early to pass judgment on the taxonomic status of Plastringe and Hartsell's organism. In view of the somewhat equivocal results, it would appear to be serologically related to *Streptococcus agalactiae*, as well as practically identical on the basis of the physiological tests. Further work will doubtless show whether it should be considered only a variety or given rank as a separate species.

#### *Streptococcus equi* (Lancefield Group C)

The Lancefield group C appears to contain three rather clearly defined biochemical groups which may justify, in the present state of our knowledge, consideration as separate species: *Streptococcus equi*, the cause of "strangles" in horses; the "animal pyogenes" or the "animal C" type; and the "human C" streptococcus which is the characteristic group C form obtained from human sources.

*Streptococcus equi* has been carefully studied by a good many specialists in comparative pathology, but is little known by bacteriologists generally; hence reference may best be made to the works of the authorities on this subject for a complete history of the organism. Jones (1919) gave a review of the subject up to the time of his work, and among recent workers may be mentioned especially Ogura (1919), Edwards (1933, 1934, 1935; Dimock and Edwards, 1933) and Evans (1936c). *Streptococcus equi* as a scientific name is generally credited to Schütz (1888), and is so credited by Bergey (1934), but Evans (1936a) states that its use by Sand and Jensen (1888) appears to be its first use as a specific name for the organism of strangles.

Those who have worked with it intimately, and should therefore be best qualified to judge, feel that *Streptococcus equi* represents a very definite and distinct physiological and clinical entity possessing characteristic and relatively constant properties. It is true that there has been much disagreement about the characteristics of *Streptococcus equi* among the older workers in this field, but from recent contributions it appears probable that most of these differences of opinion were due to a confusion with other closely related animal streptococci. For example, a number of investigators have reported lactose-fermenting streptococci, isolated from horses suffering from strangles, which were considered atypical strains of *Streptococcus equi*. Edwards (1935) dissents from this view and states that lactose-fermenting hemolytic streptococci (the "animal pyogenes" type) are sometimes found as secondary invaders; and in a recent paper Evans (1936c) concurs in the view that such lactose-fermenting strains should not be considered *Streptococcus equi*.

Its inability to ferment lactose, trehalose and sorbitol appears to serve fairly adequately for the differentiation of *Streptococcus equi* from the other group C streptococci:

<i>Streptococcus equi</i>	(lactose —, trehalose —, sorbitol —)
"Animal pyogenes"	(lactose +, trehalose —, sorbitol +)
"Human C"	(lactose ±, trehalose +, sorbitol —)

However, its identity does not rest entirely on those grounds. *Streptococcus equi* is more delicate in its nutritive requirements, very little or no growth occurring in ordinary laboratory media. The "human C" type, which on the basis of its actions on lactose and sorbitol might appear very closely related to *Streptococcus equi*, shows rather striking differences when a larger number of physiological test substances are used; the "human C" streptococcus shows greater tolerance to methylene blue (Edwards 1933, 1935), is usually actively fibrinolytic, and commonly ferments glycerol.

As some strains belonging to the Lancefield group A fail to ferment lactose, and a few strains do not attack trehalose, there is a possibility of confusing *Streptococcus pyogenes* and *Streptococcus equi* when the fermentation tests alone are depended upon,



although the possibility of such an error is not great. However, other methods in addition to the serological grouping are available for the separation of these two organisms. Following the Holman (1916) classification, in which the differentiation of *Streptococcus pyogenes* from *Streptococcus equi* rests entirely on the fermentation of lactose, many workers have reported as "*Streptococcus equi*" cultures of hemolytic streptococci from human sources, which did not ferment lactose. Evans (1936c) examined a number of such lactose-negative cultures obtained from Coburn and Pauli (1932), Fisher (1933) and Plummer (1934), and found that all of them could be readily differentiated from *Streptococcus equi* on the basis of their actions on human fibrin and trehalose. On the basis of the fermentation tests alone, the organism which could be most readily confused with *Streptococcus equi* is the "minute hemolytic streptococcus" of Long and Bliss (1934). This organism does not ferment sorbitol and may or may not ferment lactose and trehalose, and it resembles *Streptococcus equi* in growing poorly in ordinary laboratory media and in not lysing human fibrin. These two organisms belong to entirely different serological groups, and it is likely that their relative cellular and colonial "minuteness" would prevent their confusion by one who is familiar with the two organisms. Evans (1936c) reports that she has studied a number of strains of hemolytic streptococci from human sources which failed to ferment lactose, trehalose and sorbitol, "but which are otherwise very unlike *S. equi*." She also states: "In our collection of about 600 strains of hemolytic streptococci, which includes about 150 strains from animal disease sources and about 400 strains from human disease sources, there was no strain agreeing with *S. equi* from any disease other than strangles."

Other characteristics of *Streptococcus equi* which are considered somewhat unique are its high degree of virulence for white mice combined with low, or no, virulence for rabbits and guinea pigs; and its inability in general to cause agglutinin formation when injected in animals, though some "atypical" strains may have this antigenic action (Evans, 1936c).

It is possible that some of the differential characteristics which

have been claimed for *Streptococcus equi* do not rest on sound foundations; but when consideration is given to all that is known about its serological grouping, its physiological characteristics, its virulence, its very specific connection with a certain disease, and the various other peculiarities ascribed to it, there can be little doubt that it represents a unique type which may properly be designated as a species, as species are commonly defined in bacteriology.

*The "Animal Pyogenes" Streptococcus (Lancefield Group C)*

The hemolytic streptococcus belonging to the Lancefield group C, which is here designated for convenience as the "animal pyogenes," has only recently become clearly defined. Before the work of Lancefield (1933), which established its status serologically, this type had taken rather clear form as a separate entity through the works of Ogura (1929) and Edwards (1932, 1933). More recently, the physiological and serological characteristics of this organism have been further confirmed and extended by Edwards (1934, 1935), Plummer (1934, 1935) and others.

Physiologically the "animal pyogenes" is especially marked by its ability to ferment sorbitol and its inability to ferment trehalose, a combination of properties which, so far as present knowledge extends, appears to be unique among the hemolytic streptococci. Also, on the basis of the rather extensive data thus far gathered, the action of this organism on trehalose and sorbitol has proved remarkably constant.

All who have reviewed the literature on *Streptococcus equi* have cited early writers to the effect that the organism of strangles does not ferment lactose and sorbitol, whereas the "*Streptococcus pyogenes*" of other equine infections does ferment these two substances. This observation, which is contained in early treatises on *Streptococcus equi*, is credited to Holth in what appears to be an unpublished communication before 1911. (See Jones, 1919, Edwards, 1935, and Evans, 1936c.) However, it only recently became clear that the common hemolytic streptococcus of animal infections could be differentiated from the

true *Streptococcus pyogenes* of human infections by means of its action on sorbitol, though there have doubtless been valuable observations which have remained unexploited. For example, Orla-Jensen (1919) called attention to the fact that the cultures of *Streptococcus pyogenes* from human sources which he studied did not ferment sorbitol, whereas the strains obtained from animal infections did attack this substance.

In spite of the undoubted value of the trehalose and sorbitol tests in their application to hemolytic streptococci belonging to the Lancefield group C, it should again be emphasized that we are not yet acquainted with all of the important types of streptococci. For example, Minett (1935) has reported a number of strains of hemolytic, lactose-fermenting streptococci isolated from dogs, cats and ferrets, which fermented neither trehalose nor sorbitol; but it is not known whether or not these were group C streptococci, though some of them were associated with infections in lower animals. However secure present criteria may appear to be, new methods and revised definitions are apt to be necessary in connection with this and other groups of streptococci, as knowledge of the now unknown types increases.

Edwards, who has studied this streptococcus most extensively, has shown that the "animal pyogenes" is remarkably constant in other characteristics, in addition to its actions on trehalose and sorbitol and its serological grouping. All of his strains fermented lactose and salicin and produced capsules, whereas none of them fermented mannitol nor glycerol; none greened "chocolate agar," and all were inhibited by dilute methylene blue under the conditions of the test as applied by him. It should also be recalled that Lancefield (1932, 1933) found this streptococcus to be peculiarly sensitive to a particular race of bacteriophage (Clark and Clark, 1927) which she employed. Its group-specific antigen and biochemical reactions certainly make the "animal pyogenes" a distinct "species" when compared with the hemolytic streptococci outside of the Lancefield group C; and for the present at least its recognition as a definite entity of specific rank, within group C, appears justified.

Hemolytic streptococci of the "animal pyogenes" type cause

a variety of infections in lower animals. They sometimes occur in the bovine udder, occasionally causing mastitis, and may be frequently isolated from raw milk. It is now known (Dimock and Edwards, 1933) that some of the hemolytic streptococci from milk (not associated with epidemic sore throat) which were formerly considered "*Streptococcus epidemicus*" were in fact the "animal pyogenes" type. There is no evidence that this streptococcus has any significance from the standpoint of human health, and, so far as the reviewer is aware, it has not yet been isolated from human sources.

*The "Human C" Streptococcus (Lancefield Group C)*

The hemolytic streptococcus which is here designated as the "human C" type first began to take form as a distinct entity as an organism occasionally encountered from animal sources. Ogura (1929) identified this streptococcus (trehalose +, sorbitol -) as "Type B," as opposed to his "Type A" (trehalose -, sorbitol +) and *Streptococcus equi* (trehalose -, sorbitol -). As fermenting and non-fermenting strains were found with lactose, two varieties of type B were recognized: B1 (lactose -) and B2 (lactose +).

Before it became clearly established through the application of the Lancefield serological grouping method, this streptococcus was given more standing as an independent type by the work of Edwards (1932, 1933). He showed that the few cultures of this organism obtained from animal sources differed from the common "animal pyogenes" type in their respective actions on trehalose and sorbitol, and that the "Type B" streptococcus (trehalose +, sorbitol -) differed in being more tolerant to methylene blue, in not producing capsules, and in the greening of "chocolate agar." On the other hand, the fermentation tests did not effectively differentiate this ("Type B") streptococcus from *Streptococcus pyogenes* of human origin, but its greater tolerance to methylene blue separated perfectly the few cultures of this type from the true *Streptococcus pyogenes*. (Although a few of the "human hemolytic streptococci" were tolerant to methylene blue, Edwards later showed (1935) by means

of the precipitin reaction that these cultures actually belonged to the Lancefield group C.)

After the advent of the Lancefield serological technique it became apparent that whereas the usual group C hemolytic streptococcus from animal sources (the "animal pyogenes") ferments sorbitol but not trehalose, the type obtained from human beings ferments trehalose but not sorbitol. From human sources, there have now been reported some 80 or more cultures of the "human C" type of streptococcus by Lancefield and Hare (1935), Edwards (1935), Hare (1935), Plummer (1935) and Davis and Guzdar (1936); all of these strains have fermented trehalose and failed to ferment sorbitol.

Its serological grouping clearly differentiates the "human C" streptococcus from *Streptococcus pyogenes* (Lancefield group A), but on the basis of physiological tests it must be admitted that confusion is still possible. The possibility of such confusion is enhanced by the fact that the "human C" streptococcus is usually actively fibrinolytic. However, Edwards (1935) showed that all of his cultures of *Streptococcus pyogenes* (Lancefield group A) were inhibited by methylene blue, as used by him, whereas all of his "human C" streptococci grew. Edwards (1933) used a beef infusion-casein digest broth containing methylene blue in a concentration of .000025 molar. This test, as employed by Edwards, has not been used by others, but Davis and Guzdar (1936), with a 1:5,000 concentration of methylene blue in sterile milk as the test medium, confirmed Edwards' findings, though the differentiation was not perfect. In this connection, the ability to ferment glycerol may offer some supporting, though not perfect, evidence. In his early work Edwards (1932) showed that all of his five cultures of this group C streptococcus fermented glycerol, but in his later work (1935), with a somewhat larger collection, results with glycerol were not reported. Mr. Niven, in this laboratory, has examined a small number of "human C" streptococcus cultures, obtained from Dr. Lancefield, and has found them to ferment glycerol. However, it is obvious that some new methods will have to be developed before the "human C" streptococcus can be satisfac-

torily differentiated from *Streptococcus pyogenes*, except by the Lancefield serological technique.

Since both belong to serological group C, the propriety of recognizing the "human C" type as a "species" separate from the "animal pyogenes" may be questioned. Their respective actions on trehalose, sorbitol, glycerol, human fibrin, and "chocolate agar," together with their differences with respect to methylene-blue tolerance and capsule formation, give a rather imposing basis for considering these two organisms as distinct types.

The "human C" streptococcus has been obtained from the normal human nose and throat (Hare, 1935; Davis and Guzdar, 1936), vagina (Lancefield and Hare, 1935) and skin (Colebrook, Maxted and Johns, 1935). There is no evidence that this organism is of very much importance as a cause of human disease (Lancefield and Hare, 1935), but it appears that it may sometimes cause erysipelas (Hare, 1935). Plummer (1935) studied two cultures from puerperal fever and three from erysipelas. With respect to animals, Edwards (1935) states that this streptococcus is of low virulence and, when present in severe infections, is usually associated with other streptococci.

*The "Minute Hemolytic Streptococcus" of Long and Bliss (Lancefield Group F)*

The "minute hemolytic streptococcus" discovered by Long and Bliss (1934) represents a new species of streptococcus of undoubted authenticity. In blood agar plates this organism produces extremely small "pin-point" colonies, frequently barely visible, but surrounded by a definite zone of true hemolysis ranging from a little more than 0.5 to about 1.5 mm. after 48 hours.

Long and Bliss based this new species not only on its unique morphology, colony formation, and fermentation reactions, but also reported preliminary serological investigations which showed that this organism did not belong to any of the then established Lancefield groups (A, B, C, D and E). Shortly afterward,

Lancefield and Hare (1935) identified this organism from the human vagina, and designated it as serological group F.

Although the "minute hemolytic streptococcus" gives a rather characteristic "pattern" on the fermentation tests, some diversity is found among different strains on some of the more commonly used test substances. Lactose, trehalose and salicin may or may not be fermented, but a vast majority of strains ferment trehalose and salicin. Relatively few of the strains reported by Long and Bliss fermented lactose, whereas a majority of those reported by Hare (1935), Hare and Maxted (1935) and Lancefield and Hare (1935) did attack this substance. The group F streptococcus may or may not produce "soluble hemolysin" when subjected to the conventional test (Hare and Maxted, 1935), but Long and Bliss (1937a) have shown that this substance is abundantly produced with the application of appropriate methods. Aside from its serological grouping, the identity of the "minute hemolytic streptococcus" is perhaps established more firmly by its morphological and colonial characteristics than by its physiological properties. In this connection, it is desirable to give a few direct quotations from Long and Bliss (1934), who have carefully studied these features of their organism.

In films made from cultures in liquid medium and stained by Gram's method the organisms appear as minute cocci occurring singly, in pairs, in short chains and in small and large masses. The individual coccus is one-half to two-thirds the size of the ordinary *beta* hemolytic streptococcus and it stains indifferently with Gram, some strains being strongly Gram-positive while others are Gram-negative.

When the areas of hemolysis first become visible the colony cannot be distinguished by means of the unaided eye, and resort must be had to the use of the low power of an ordinary microscope. At this stage of development, the colony appears as a small, finely granular, roughly circular object ranging in size from 18 to 30 microns and surrounded by a relatively large area of true *beta* type hemolysis. Occasionally the colonies appear to be wrinkled and crenated or they may have a curious tetradic appearance. Rarely have the colonies been oval in the primary culture. By the end of 48 hours incubation they are visible to the naked eye, although, in certain instances, 96 hours of incubation

were required before they were visible. In the first stages of development the ratio of the diameter of the area of the hemolysis to the diameter of the colony is roughly from 4 to 1, to 10 to 1. With further incubation this ratio decreases so that by the end of 48 hours the ratio is generally 3 or 4 to 1. . . . Ordinary *beta* hemolytic streptococci from human sources have a ratio of 3 or 4 to 1 from the time the colonies are first visible and preserve this ratio throughout the period of incubation.

Bliss (1937) has investigated the serological characteristics of the group F "minute" streptococci. By means of the slide agglutination technique four serological types, within group F, were established.

Long and Bliss (1934) and Long, Bliss and Walcott (1934) have isolated the "minute hemolytic streptococcus" from the throats of normal people and from those suffering with a variety of diseases, especially glomerular nephritis and rheumatic fever. In a few cases the organism was recovered in pure culture from pus removed from inflamed sinuses and abscesses. These investigators have been conservative in their conclusions and the importance of this streptococcus as a cause of human disease is still held to be problematical. However, Bliss (1937) has referred to a paper now in press (Long and Bliss, 1937b) which reports several cases in which group F streptococci appeared to be the primary cause of disease.

Regardless of its possible importance as an occasional invader of the human body, there is no doubt that the "minute hemolytic streptococcus" commonly occurs as a harmless human parasite. Aside from its occurrence in the normal throat, this streptococcus has been obtained from the human vagina (Lancefield and Hare, 1935), skin (Colebrook, Maxted and Johns, 1935) and feces (Hare and Maxted, 1935).

#### *The Streptococci Belonging to the Lancefield Group G*

The streptococci of this group were discovered through the work of Long and Bliss (1934) and were noted by Lancefield and Hare (1935) as the serological group G. In their paper dealing with the physiological classification of "minute hemolytic streptococci," Long and Bliss (1934) segregated as "group II"



the organisms which fermented lactose. Correlated with the fermentation of lactose, in most of the cultures, was a somewhat wide zone of hemolysis surrounding the colonies in blood agar plates. Preliminary studies of the antigenic structure of these organisms had shown that this type was serologically different from the other "minutes" (group F), and later studies (Bliss, 1937) showed that these somewhat larger "minutes," which showed a wider zone of hemolysis, belonged to the Lancefield group G, which group in the meantime had been established by Lancefield and Hare (1935) on the basis of cultures isolated from the human vagina.

From an inspection of the characteristics ascribed to group G streptococci in table 2, it would appear that this group is made up of diverse physiological types. Physiological studies of group G cultures indicate that this group, like group C, contains at least two, and more probably three, biological types which may eventually be recognized with some degree of accuracy by the use of biochemical tests. In this apparently heterogeneous group there occurs one clearly defined and homogeneous physiological type which future work may indicate should be recognized as a distinct variety, or even species, within group G. This type is the one which Long and Bliss (1934) included among their "minute hemolytic streptococci." As the more recent work of Bliss (1937) has shown, the members of group G which Long and Bliss had previously classified as "minute" streptococci all belong to serological type I, whereas the strains which were considered as beta hemolytic streptococci of ordinary size do not belong to this serological type.

Aside from the "minute" or type I variety, the other members of the Lancefield group G appear to represent rather diverse physiological types. Among these are found strains which in physiological characteristics, including strong fibrinolytic action, appear to be identical with *Streptococcus pyogenes*. On the other hand, some strains belonging to group G differ widely from *Streptococcus pyogenes* in that they are able to attack a variety of substances including glycerol and starch, sometimes even fermenting weakly inulin and the pentose sugars. This type, which may or

may not be fibrinolytic, also differs widely from *Streptococcus pyogenes* in being more tolerant to methylene blue, in having stronger reducing action, and in the ability of some strains to grow at 45°C.

From studies made in our laboratories with cultures furnished by Drs. Bliss and Lancefield, as well as additional strains isolated from human feces, it appears that the "minute" or type I (Bliss, 1937) group G organisms represent a rather clearly defined physiological variety of streptococcus. Although similar to *Streptococcus pyogenes* with the conventional tests, all of the type I strains which we have thus procured have been found to ferment raffinose, curdle milk, and to be devoid of strong fibrinolytic action, thus being differentiated physiologically from *Streptococcus pyogenes*. In making these statements it should be emphatically asserted that no claim is made that serologically types cut "species" lines in group G. It is not certain, nor even highly probable, that all strains which have these physiological characteristics will prove to belong to type I. It just so happens that, among the limited number of cultures which have been studied, the members of serological type I conform to a rather definite physiological pattern which differs from that of the non-type-I strains.

It is perhaps proper to point out similarities between newly discovered organisms and types which have been previously described. Attention is therefore called to the likeness of the type I group G streptococcus to the *Streptococcus anginosus* of Andrewes and Horder (1906). Before entering this discussion, however, it should be made clear that different criteria have been used in the identification of this organism. In the Holman (1916) classification, *Streptococcus anginosus* is described simply as a hemolytic, lactose-fermenting streptococcus which ferments neither mannitol nor salicin. *Streptococcus pyogenes* is given the same characteristics except that salicin is fermented, the differentiation of these two organisms resting wholly on the salicin reaction. We are not concerned with which is the "correct" definition; only that they are different. The present comparison is with the type as given by Andrewes and Horder.

Andrewes and Horder (1906) described as *Streptococcus anginosus* a hemolytic streptococcus which they isolated from normal human throats, and frequently also from cases of scarlet fever and other forms of sore throat. *Streptococcus anginosus* was differentiated from *Streptococcus pyogenes* by its ability to curdle milk, ferment raffinose, and produce a stronger reducing action in neutral red cultures. They also noted variants from the type, some of which did not ferment raffinose, while others fermented inulin in addition to raffinose. Fermenting and non-fermenting strains were found with salicin, the majority failing to attack this substance. There can be little doubt that *Streptococcus anginosus* as described by Andrewes and Horder represents a distinct type from *Streptococcus pyogenes*; and since the other long-established species of pathogenic, hemolytic streptococci, such as *Streptococcus mastitidis* and *Streptococcus equi*, never ferment raffinose, *Streptococcus anginosus* (Andrewes and Horder) would seem to represent an authentic type or species. This organism has apparently been lost during the past thirty years. The reason for this loss is not far to seek; it was caused by concentrating on a few reactions and at the same time discarding tests of merit for the portrayal of the characteristics of streptococci. Hence, the selective raffinose fermentation was dropped, the old-fashioned milk test was eliminated, and tests for the relative reducing abilities of streptococci were discontinued. Thus the tests which were especially advocated by Andrewes and Horder for the recognition of their newly discovered species fell by the wayside.

Again it should be emphasized that aside from those organisms of the Lancefield group G which bear a relationship to *Streptococcus anginosus*, and represent an apparently closely related and homogeneous type, there occur other types which do not appear very closely related either to the *Streptococcus anginosus* type or to each other.

The hemolytic streptococci belonging to the Lancefield group G are widely distributed. They have been obtained from the normal human throat and nose, vagina, skin and feces. Certain types belonging to this group have also been obtained from

normal animal throats and from animal infections, their occurrence in the latter being possibly only as secondary invaders. (Long and Bliss, 1934; Lancefield and Hare, 1935; Hare, 1935; Hare and Maxted, 1935; Colebrook, Maxted and Johns, 1935; Davis and Guzdar, 1935.) There is no present reason for believing that the group G streptococci are of much importance as potential producers of human disease. Lancefield and Hare (1935) obtained one strain from a severe case of puerperal infection, but in this case there was also a heavy staphylococcus infection. In the light of present information, it seems probable that group G streptococci are only of occasional, if any, importance from the standpoint of human health.

*The Lancefield Group E Streptococcus*

The group E hemolytic streptococcus was established by Lancefield (1933) on the basis of three cultures which had been obtained from milk. As only a few strains of the group E streptococcus have been serologically identified since the work of Lancefield, it is not known how commonly this type occurs; thus far, it has been reported only from the bovine udder and milk. The few cultures which had been subjected to detailed study show considerable diversity in respect to the fermentation tests and the identity of this streptococcus as yet rests entirely on the Lancefield serological grouping. In view of the limited knowledge of this group, Dr. Lancefield's brief statement concerning it is reproduced.

Group E comprised three strains isolated by Dr. J. H. Brown from certified milk. They were members of Groups 3 and 6 described by Brown, Frost, and Shaw (1926). On preliminary examination they were thought to be members of Group C of this series because one of them, K 131, showed some cross-reaction with Group C antisera and because all three were very hemolytic on blood agar plates and also markedly susceptible to streptococcus bacteriophage. Further work showed that the precipitin reaction of Strain K 131 with Group C antisera was a minor one, not exhibited by the other two strains, and that antisera prepared against these three strains showed strong precipitin reactions with extracts of all three.

The antiserum from one rabbit immunized with Strain K 129 showed traces of cross-reactions with almost all extracts tested. This was not evident with the antiserum from another rabbit; hence it was felt that this difference had to do with an individual rabbit variation rather than with antigens contained in this strain.

The cultural characteristics of this group were the following: the final pH attained in dextrose broth was 4.6 to 4.8; sodium hippurate was not hydrolyzed; methylene blue milk was not reduced; growth was not obtained on bile blood agar even when the concentration of bile was only 10 per cent; both trehalose and sorbitol were fermented; and the streptococcus bacteriophage caused rapid lysis of all three strains. It is not known how extensively this group is distributed, nor whether it is composed entirely of strains derived from milk, although the three classified here were isolated from certified milk.

It will be noted that group E streptococci ferment both trehalose and sorbitol, which is unique among the established species of the hemolytic streptococci, outside of the enterococcus division; it should be recognized, however, that not sufficient strains have been studied to establish these properties as constant characteristics of the group. It is probable that to this group belong at least a portion of the hemolytic streptococci reported from milk, which ferment both trehalose and sorbitol (Minett, 1935). Among the few strains of group E streptococci thus far studied, there have been cultures which fermented both mannitol and salicin, and others which fermented neither of these substances. It is therefore likely that some of the hemolytic streptococci from milk which have been reported under the name of *Streptococcus infrequens* (Frost, Gumm and Thomas, 1927; Minett and Stableforth, 1934) were members of this group; and it is also possible that some of the strains designated as *Streptococcus asalignus* (Frost, Gumm and Thomas, 1927) were group E streptococci, though most of these were probably members of group B.

The group E cultures reported by Lancefield were strongly hemolytic, but Plastringe and Hartsell (1937) have reported strains obtained from the bovine udder which they classified as weakly hemolytic. An important point which supplements

the Lancefield serological classification in establishing the group E streptococcus as an authentic species, is the work of Todd (1934) which showed that the streptolysin produced by this organism has characteristic properties, its acid stability being especially outstanding.

*The Group H Streptococcus of Hare*

By means of the Lancefield serological method Hare (1935) segregated as a new group (group H) hemolytic streptococci obtained from the human nose and throat. Hare and Maxted (1935) also recovered two group H strains from human feces. These cultures did not lyse human fibrin, hydrolyze sodium hippurate, grow on 40 per cent bile blood agar, nor produce "soluble hemolysin." Hare studied the fermentation reactions of his cultures with lactose, trehalose, mannitol, sorbitol and salicin. All of the strains fermented salicin; twenty-four of the 25 cultures failed to ferment mannitol and sorbitol, but one culture fermented both of these substances; a majority of the cultures fermented lactose (22 +, 3 -) and trehalose (21 +, 4 -). It is evident that the members of this group show some diversity in the fermentation tests. Since the group H streptococcus was discovered by Hare and the identity of the group rests on his work, it is desirable to quote Hare's description of this organism.

This is a new group not hitherto described. The strains composing it give very regular biochemical reactions and have on the whole very distinctive appearances on blood agar. Serologically they are quite distinct from the other groups, and there can be little doubt that they do form a distinct group of haemolytic streptococci. The colonies tend to be somewhat smaller than those of group A (0.7-0.9 mm. as against 0.8-1.3 mm.), but they are usually of matt surface, and at first of greenish colour although tending to blacken after continued incubation. The colonies themselves are hard, almost gritty in consistency and adhere closely to the medium so that they are difficult to dislodge. The area of haemolysis produced is usually much the same as that of group A strains, but in the 8 per cent. horse blood agar generally employed it is seldom quite complete; in Brown's 1.5 per cent agar with

5 per cent of horse blood it is, however, quite complete. With continued incubation (48 hours or more) the haemolysis, particularly on 8 per cent agar, becomes alpha-prime in character, i.e. the area in the immediate neighbourhood of the colony becomes green and is surrounded by a zone of clear haemolysis. Only one strain has failed to give this appearance. On boiled blood agar, such strains give definite areas of green methaemoglobin, in which respect they differ from the strains of groups E and F and the majority of group K.

Biochemically these strains are unable to form soluble haemolysin, they give a pH in 1 per cent glucose broth very slightly below that of group A strains, they have no action on hippurate or fibrin and they have the same fermentation reactions as group A strains.

It is probable that they are the same as the strains isolated from the sputum of tuberculous patients by Cumming (1927) and named by him "pseudohaemolytic streptococci" because they resembled closely strains from infections but differed in being unable to form soluble haemolysin. I have been unable to confirm his statement that such strains invariably lose their ability to give haemolysis on blood agar after more than four subcultures, although several strains have shown a tendency to throw off non-haemolytic colonies. It is improbable that strains belonging to this group ever cause serious human infections; but seeing that they are relatively common in throat swabs and resemble group A strains superficially, it would seem that they have frequently been looked upon by previous workers as identical with pathogenic strains.

The more extended description of the group H streptococcus contained in tables 1 and 2, covering characteristics not determined by Hare, is based upon the examination of only two of Dr. Hare's cultures which were obtained for this study from Dr. Lancefield. If, however, these two cultures are representative of the group as a whole, it would appear from such characteristics as its ability to grow at 45°C., its thermal resistance, and its ability to ferment raffinose that this streptococcus differs radically from *Streptococcus pyogenes*.

#### *The Group K of Hare*

Hare (1935) obtained eight cultures of streptococci from the human nose and throat which appeared to form a homogeneous and distinct serological group by the Lancefield precipitin

method, and for which he has tentatively suggested designation as group K. As only these eight cultures of group K have been reported, and especially since Hare has suggested this group only provisionally, it has not been included in the tables of this review as representing a more or less established "species." Also, from Hare's description (and from the study of a few of Dr. Hare's cultures) some doubt is felt as to whether most bacteriologists would classify these organisms as truly hemolytic streptococci. Aside from his tabular material, Hare makes the following brief statement concerning this group:

This is a new and provisional group of which only eight representatives have been isolated. The colonies tend to be about the same size as those of group A but they are moist and transparent, with crenated edges. The area of haemolysis produced in 8 per cent horse blood agar is usually small and incomplete, being seldom more than 3 mm. in diameter, although it may be larger and quite complete in Brown's 5 per cent agar. An alpha-prime appearance is not produced.

Biochemically, these strains resemble group H strains in their inability to form soluble haemolysin, to dissolve fibrin or to act on hippurate. Their pH in 1 per cent glucose broth is, however, higher, being 5.1 to 5.4, and seven out of the eight strains were unable to ferment trehalose. There is thus some justification for looking on these strains as a separate group. I have never encountered strains of this group in disease processes nor in other situations than the human throat.

With the five fermentation test substances used, Hare found that lactose was fermented, and mannitol and sorbitol were not fermented. While fermenting and non-fermenting strains were found with trehalose and salicin, six of the eight cultures fermented salicin but only one attacked trehalose. They did not grow on blood agar containing 40 per cent bile.

#### THE VIRIDANS STREPTOCOCCI

The "viridans" group, as here defined, is probably the least satisfactory of the divisions of the streptococci, and in all probability it is by far the most incomplete from the standpoint of the number of known types which may now be assigned to it. The fairly clearly defined types which are included in this division



probably represent only a fraction of the non-hemolytic streptococci (alpha and gamma types) which fall outside of the lactic and enterococcus groups. Although the types recognized at present have a number of basic characteristics in common, it is highly improbable that all existing related non-hemolytic streptococci will be found to fit into this physiological pattern. The "viridans division" is therefore to be considered as probably having only a temporary usefulness in the present state of our ignorance of the streptococci belonging to this general group.

A great variety of human ailments have been ascribed to "*Streptococcus viridans*" and to "indifferent streptococci," and many specific names have been applied to organisms which were thought to have etiological importance in connection with those diseases. Although these inadequately described organisms are generally considered to be related to *Streptococcus salivarius*, how many of them belong to the established species of the non-hemolytic streptococci cannot be stated at the present time; and until some one takes the trouble to study them carefully we need not try to speculate upon their taxonomic relationships. In this connection, however, it may be pertinent to mention that according to Hucker (1937b), whose results have not yet been published, practically all of the strains contained in a collection of nearly 200 cultures of "*Streptococcus viridans*" obtained from clinical sources were found upon detailed physiological study to be either *Streptococcus salivarius* or *Streptococcus fecalis*, the larger portion belonging to the former species.

Serological methods have not proved useful tools for the classification of these streptococci. The specific carbohydrate appears to be type specific in the viridans streptococci (Lancefield, 1925a, 1925b; Hitchcock, 1924b), instead of group or species specific as in the case of the hemolytic forms. Hence they form a serologically heterogeneous group which is not amenable, for purposes of taxonomy, to the invaluable Lancefield technique. This is also in line with the experience gained with other serological methods; from the beginning of such studies the diversity of the non-hemolytic streptococci has usually been emphasized as compared with the relative homogeneity of the hemolytic

types. (Kligler, 1915; Krumwiede and Valentine, 1916; Kinsella and Swift, 1917, 1918; Howell, 1918; Havens, 1919; Dochez, Avery and Lancefield, 1919; Clawson, 1920; Herrold, 1922; Norton, 1923; Crow, 1933.) For example, Gordon (1922) found that of 131 cultures of *Streptococcus pyogenes*, 125 were of the same serological type as revealed by agglutination and agglutinin absorption tests; whereas with 16 cultures of *Streptococcus salivarius*, at least 12 different types were found by the same methods.

In this division of the streptococci, therefore, the classification must at present rest entirely on physiological characteristics.

TABLE 3

*The viridans streptococci: additional characteristics*

SPECIES OR VARIETY	GROWTH IN 2 PER CENT NaCl	STARCH HYDROLYZED	SODIUM HIPPOURATE HYDROLYZED	ESCULIN SPLIT	GELATIN LIQUEFIED	MILK CURDLED	FINAL pH IN GLUCOSE BROTH	ACID PRODUCED FROM									
								Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol
<i>S. salivarius</i> .....	+	-	-	±	-	±	5.4-4.0	-	+	+	+	±	±	±	-	-	±
<i>S. equinus</i> .....	+	±	-	+	-	-	4.5-4.0	-	+	±	±	±	±	±	-	-	±
<i>S. bovis</i> .....	+	+	-	+	-	±	4.5-4.0	±	+	+	+	±	±	±	-	±	+
Varieties of <i>S.</i> <i>bovis</i> .....	+	-	*	+	-	±	4.5-4.0	-*	+	+	+	±	±	±	-	±	+
<i>S. thermophilus</i> ...	-	-	*	-	-	+	4.5-4.0	-*	-*	+	+	±	±	-	-	-	-

\* See table 1.

Although these appear to be fairly satisfactory for the differentiation of at least a few types among the viridans streptococci, one need only reflect upon the revisions made necessary in our ideas about the groups of hemolytic streptococci with the advent of the Lancefield grouping, to realize what may happen at any time to the most carefully constructed creations of present day pattern builders.

As has been previously pointed out and as is shown in table 1, the recognized types among the viridans streptococci show certain characteristics in common which justify their inclusion in

a separate division or group of the streptococci. The combination of characteristics which appear to justify such a grouping is the inability to cause true beta hemolysis of blood, the possession of high minimum and relatively high maximum temperatures of growth, weak reducing action, a limited tolerance to methylene blue, salt and alkali, and the inability to produce ammonia from peptone. In table 3 are given the additional characteristics of the members of this group.

*Streptococcus salivarius*

Andrewes and Horder (1906) gave the name of *Streptococcus salivarius* to the organism which they found to be the predominating streptococcus of the human throat. This organism was also sometimes found, probably only surviving rather than growing, in feces, and it was encountered in certain infections, especially endocarditis. Streptococci of this general type had of course been studied before the work of Andrewes and Horder; there can be little doubt, for example, that some of the organisms studied by Schottmüller (1903) under the name of *Streptococcus mitior* were the same as *Streptococcus salivarius*; but since Andrewes and Horder were the first to describe this organism with sufficient detail to make its identity reasonably clear, the name *Streptococcus salivarius* appears to be the one which should be given priority for organisms of this general type.

The outstanding characteristics which Andrewes and Horder ascribed to *Streptococcus salivarius* may be briefly stated as follows: Blood is not hemolyzed; milk is acidulated and curdled; neutral red is usually reduced; lactose and sucrose are fermented; raffinose is usually fermented, but inulin less frequently; the glucosides, salicin and coniferin, may or may not be fermented; but mannitol is never fermented. During a decade or more following the work of Andrewes and Horder, the tendency among bacteriologists was to use fewer tests in the characterization of streptococci; such characteristics as action on milk, reducing properties, and the fermentation of raffinose were largely dropped; and hence *Streptococcus salivarius* to some extent lost its identity in terms of its characterization by Andrewes and

Horder. Because of the various criteria which have been subsequently used by bacteriologists in the description of *Streptococcus salivarius*, it is pertinent to quote Andrewes and Horder (1906) on the subject: "It clots milk almost always and in its typical form reduces neutral red, though variants occur which fail to do this. The characteristic fermentation reactions are saccharose, lactose, and raffinose, the last named less constantly than the first two. The glucoside reactions may be added, and rarely inulin." Again they state: "The common positive chemical reactions are clotting of milk, reduction of neutral red, and acid formation with saccharose, lactose, and often raffinose, but not with mannite. Reactions with the glucosides are often added. . . ."

With the virtual discontinuance of some of the tests emphasized by Andrewes and Horder, *Streptococcus salivarius* has been described in various ways. In one system, this organism is defined as a non-hemolytic streptococcus which ferments lactose but does not ferment mannitol nor salicin. In another classification, which retains the raffinose reaction, *Streptococcus salivarius* is identified simply as non-hemolytic and fermenting raffinose but not mannitol.

Andrewes and Horder described as a separate species *Streptococcus mitis*, an organism found principally in the human mouth, which was very closely related to *Streptococcus salivarius* and which might, in fact, be considered simply as a variety of the latter species. *Streptococcus mitis* was described as not curdling milk, having only slight reducing action on neutral red, and usually not fermenting raffinose. Otherwise, its characteristics are the same as those of *Streptococcus salivarius*. Andrewes and Horder described *Streptococcus mitis* in these terms: "It is a short-chained form . . . and it gives a marked acid reaction in milk, though no clotting. . . . Its typical positive reactions on Gordon's tests are saccharose and lactose with or without the glucosides. . . . Such forms may with fair propriety be regarded as variants (of *Streptococcus salivarius*) in which the power of clotting milk has been suppressed."

It is obvious from the foregoing quotation that Andrewes and

Horder considered *Streptococcus mitis* as being so closely related to *Streptococcus salivarius* that its validity as a species was somewhat in doubt; and in recent years most bacteriologists have not attempted to differentiate such a species from *Streptococcus salivarius*. However, in the useful and much-followed Holman (1916) classification, *Streptococcus mitis* is defined as being non-hemolytic, fermenting lactose and salicin, but not fermenting mannitol. In this classification, *Streptococcus salivarius* and *Streptococcus mitis* have the same characteristics with the exception that *Streptococcus mitis* ferments salicin while *Streptococcus salivarius* does not.

In a recent investigation of this group of streptococci Safford, Sherman and Hodge (1937) applied a broad list of physiological tests to the study of these organisms. These investigators concluded that the type of streptococcus which occurs in largest numbers in the human throat is one which follows closely the description given by Andrewes and Horder for *Streptococcus salivarius*. In blood agar the reaction varies from a complete gamma type in a few cultures to a marked alpha reaction in others, a weak alpha reaction being most characteristic. What was considered the typical form of *Streptococcus salivarius* by these investigators is an active acid-producing organism which gives a final pH value in glucose broth of 4.4 to 4.0, and which acidulates and curdles milk promptly. Although there is no reduction of litmus in milk cultures before curdling takes place, a marked and almost complete reduction occurs after coagulation. A majority of cultures of this type ferment raffinose and also inulin, though the fermentation of inulin is less general.

Although the description given in the above paragraph is considered accurate for *Streptococcus salivarius* in what Andrewes and Horder might call "its typical form," all the non-hemolytic streptococci from the human throat which belong to this same general group do not fall into such a rigid physiological pattern. In a collection of such organisms there are also found many cultures which appear to agree in a general way with the type described by Andrewes and Horder as *Streptococcus mitis*. No rigid line can be drawn between this somewhat indefinite variety

and the typical *Streptococcus salivarius*. However, the mitis type is characterized by a weak acid-producing power; milk is not coagulated, and in glucose broth final pH values of 5.3 to 4.7 are attained. These weak acid-producing forms usually do not ferment raffinose or inulin, and there also appears a tendency to produce a stronger or more definitely alpha type of action on blood than is true of the more typical form of *Streptococcus salivarius*. Although there appear to be some more or less correlated divergencies from type, the differences between the so-called *Streptococcus mitis* and the characteristic form of *Streptococcus salivarius* are quantitative ones without any apparently rigid lines of demarcation. Under these conditions, there does not appear to be a satisfactory basis for the establishment of *Streptococcus mitis* as a separate species at the present time. However, it should be recognized that the more or less closely related non-hemolytic streptococci of the human throat undoubtedly form a rather complex group, which probably will be found to contain more than one species when more penetrating methods have been developed for the study of these organisms.

### *Streptococcus equinus*

Non-hemolytic streptococci which do not ferment lactose were first observed as contaminants from air. Andrewes and Horder (1906) suspected that these organisms might come from horse dung, which at that time made up a large part of the organic pollution in the air of cities. An investigation of fresh horse feces showed that this type of organism is the principal streptococcus found in the intestine of the horse, usually, in fact, constituting the majority of the total bacterial population of such material.

This organism, which they named *Streptococcus equinus*, was described by Andrewes and Horder as being devoid of pathogenic properties, non-hemolytic, and having the following additional characteristics: milk is not coagulated; there is little or no reducing action on neutral red; sucrose, salicin and coniferin are usually fermented; lactose and mannitol are not fermented; raffinose and inulin are not attacked as a rule, but a number of

variant types which ferment these substances were found. Andrewes and Horder also pointed out the important fact that *Streptococcus equinus* has a high minimum temperature of growth, evidenced by little or no growth in gelatin cultures at 20°C. They considered horse dung the chief source of the organism but thought that it might occur in the intestines of other herbivora. They did not succeed in obtaining it from the intestines of certain carnivora examined.

Winslow and Palmer (1910) likewise found *Streptococcus equinus* the predominating organism in the intestine of the horse and also reported the finding of similar non-lactose-fermenting streptococci in bovine and human feces. Fuller and Armstrong (1913), Floyd and Wolbach (1914), Broadhurst (1915), Holman (1916), Blake (1917), Arnold (1920), and many others, have reported from various sources streptococci, which did not ferment lactose, under the name of *Streptococcus equinus*. Aside from animal feces, such streptococci have been obtained frequently from the human mouth and also occasionally from the urine and from infections, though these organisms have not been clearly implicated as causative agents in such infections.

Whether or not the streptococci failing to ferment lactose and which have been frequently obtained from human sources, especially the throat, are in fact the same as the true *Streptococcus equinus* of equine origin cannot be stated on the basis of present knowledge. In recent years, the only requirements for an organism to be classified as *Streptococcus equinus*, or at least as a member of the "equinus group," are that it be a non-hemolytic streptococcus which fails to ferment lactose and mannitol. Hodge and Sherman (1937) emphasized some of the little used tests of Andrewes and Horder, such as the minimum temperature of growth, and added a number of other characteristics in their description of this organism. These investigators thought that the general "pattern" of reactions given by this organism, aside from its inability to ferment lactose, was sufficiently clear to mark it as a type.

As may be seen from table 3, *Streptococcus equinus* shows points of close relationship to *Streptococcus salivarius*, on the

one hand, and to *Streptococcus bovis* on the other; in a number of respects it might be considered as falling between these two species. Hodge and Sherman commented on the relationship of *Streptococcus equinus* to *Streptococcus bovis* and stated that although they suspected that *Streptococcus equinus* might hydrolyze starch, they found no evidence of this action when the cultures were tested in starch agar by the dilution, poured-plate method. However, in more recent tests with a few typical cultures of *Streptococcus equinus* obtained from horse feces, it has been found that this organism may hydrolyze starch when a more favorable medium is used and the plates are inoculated by the streak method. If the hydrolysis of starch should prove to be a general characteristic of the true *Streptococcus equinus*, this method might prove useful in the differentiation of this organism from aberrant strains of closely related types which fail to ferment lactose.

In view of the unique characteristics of *Streptococcus equinus* and its close relationship to certain other species, a few comments are needed on its physiological characteristics which are not clear from the tabular material presented. An outstanding feature is its high minimum temperature of growth of about 20°C. *Streptococcus equinus* grows poorly in milk, even with added glucose, and it has very slight reducing action. Although sucrose, raffinose, inulin and salicin may or may not be fermented, sucrose and salicin are fermented by a large majority of strains, whereas raffinose and inulin are attacked by a relatively small percentage of cultures.

Compared with *Streptococcus salivarius*, *Streptococcus equinus* appears to have a somewhat higher maximum temperature of growth and a slightly higher thermal resistance, and less frequently ferments raffinose and inulin. On the other hand, *Streptococcus bovis* has a higher thermal death point than *Streptococcus equinus*, more frequently ferments raffinose and inulin, and usually ferments arabinose.

In the Holman (1916) classification, which is based on four characters, strains of the "equinus group" which do not ferment salicin are designated as *Streptococcus ignavus*. In this system,



the difference between *Streptococcus equinus* and *Streptococcus ignavus* rests entirely on the salicin reaction, both species being otherwise described as non-hemolytic and failing to ferment lactose and mannitol.

*Streptococcus bovis*

Winslow and Palmer (1910) and Fuller and Armstrong (1913) observed that the prevailing type of streptococcus in the feces of the cow ferments raffinose, while the predominating forms from the intestines of man and the horse do not have this property. As their studies were largely limited to the fermentation tests, Fuller and Armstrong considered this organism, on the basis of the raffinose reaction, as identical with the *Streptococcus salivarius* of the human throat.

*Streptococcus bovis* was described as a new species and named by Orla-Jensen (1919), who obtained it from cow feces and from milks which had been heated or incubated at high temperatures. Although obtained from a number of sources, this streptococcus was recognized as having its habitat in the bovine alimentary tract. Although Orla-Jensen did not study *Streptococcus salivarius*, his description of *Streptococcus bovis* is nevertheless adequate to indicate strongly, if not thoroughly to establish, its integrity as an independent species. Aside from being somewhat unusual with respect to the fermentation tests, *Streptococcus bovis* was observed to have rather marked thermal resistance and to grow at relatively high temperatures. With respect to substances which are not generally fermented by streptococci, *Streptococcus bovis* was found to be an active fermenter of arabinose, raffinose, starch, and usually inulin.

Ayers and Mudge (1923) found *Streptococcus bovis* to be the predominating streptococcal form in the mouths and intestines of cows. Aside from the fermentation tests, these investigators studied *Streptococcus bovis* along somewhat different lines and added further information concerning its characteristics, such as its inability to produce ammonia from peptone, its weak reducing action, its action on sodium hippurate, blood, etc. Sherman and Stark (1931) also studied *Streptococcus bovis*, combining the methods of Orla-Jensen and of Ayers and Mudge,

and confirmed the results of these investigators. Sherman and Stark also paid especial attention to the temperature limits of growth, and noted as a particular characteristic of *Streptococcus bovis* its ability to hydrolyze starch actively when tested by the starch agar method.

Although the relationship between *Streptococcus bovis* and *Streptococcus salivarius* is obviously close, an inspection of the data given in tables 1 and 3 indicates a number of divergencies between these two types. Compared with *Streptococcus salivarius*, *Streptococcus bovis* has a somewhat higher maximum temperature of growth, a distinctly greater thermal resistance, hydrolyzes starch, and usually ferments arabinose; some strains ferment mannitol. Those who have worked intimately with these two groups of organisms feel that they are distinct types, but it must be admitted that convenient and decisive tests for their differentiation are still needed. The confusion between *Streptococcus bovis* and *Streptococcus salivarius* becomes greater in the case of the non-starch-hydrolyzing varieties of the "bovis group."

*Streptococcus bovis* has been isolated from human feces, and what appear to be simply varieties of this organism, which do not hydrolyze starch, are commonly found in the human intestine. The question as to whether or not *Streptococcus bovis*, as in the case of a number of other non-pathogenic streptococci, may occasionally be found in human infections, cannot be answered as the methods ordinarily used in the study of non-hemolytic streptococci from clinical sources are not adequate for the identification of this organism. As will be noted later, some strains of the so-called "Bargen streptococcus," thought by some to be associated with ulcerative colitis, have proved to be starch-hydrolyzing streptococci which appear to be identical with *Streptococcus bovis*.

*Varieties of Streptococcus bovis: Streptococcus inulinaceus and the "Bargen Streptococcus"*

*Streptococcus inulinaceus* was so named by Orla-Jensen (1919) who described this type as an independent species, though noting that it was very closely related to *Streptococcus bovis* and

might be considered as a variety of that species. The type described as *Streptococcus inulinaceus* does not ferment starch, or ferments it only weakly, and does not ferment arabinose, substances which are actively fermented by the typical *Streptococcus bovis*. Sherman and Stark (1931) also found that the members of the "bovis group" which did not hydrolyze starch also failed to ferment arabinose, while the fermentation of arabinose appeared to be perfectly correlated with the hydrolysis of starch in their strains of the typical *Streptococcus bovis*; their results therefore tended to add some validity to *Streptococcus inulinaceus* as an independent type, though it was suggested that it probably should be considered as a variety of *Streptococcus bovis*.

In a recent study of the *Streptococcus bovis* group (Stark and Sherman, 1937) even less grounds have been found for considering *Streptococcus inulinaceus* as a separate species. A considerable proportion of starch-hydrolyzing strains have been obtained which do not ferment arabinose, thus spoiling the correlation which was indicated by previous work. Earlier work had also indicated that the so-called *Streptococcus inulinaceus* is especially characteristic of the bovine throat, while *Streptococcus bovis* is the predominating type in the intestine; but this has not been confirmed by more recent findings, the typical starch-hydrolyzing *Streptococcus bovis* being the prevailing form in both the mouth and the feces. Unless very fine lines are to be drawn in the establishment of species in this group, there would not appear to be sufficiently substantial grounds for considering the non-starch-hydrolyzing type as more than a variety of *Streptococcus bovis*.

What has become colloquially known as the "Bergen streptococcus" is a form which Bergen (1924, 1930) considers as having a causative relationship to ulcerative colitis. For our present purposes it is not pertinent to review the considerable body of literature, chiefly clinical, which has grown up around this moot question. However, reference may be made to the recent papers of Torrey and Montu (1934, 1936) which contain reports on studies of the organism, as well as citations to the literature. The identity of the "Bergen streptococcus" has remained ob-

scure. Some have identified the organism simply as a somewhat heat-tolerant diplococcus or short-chained streptococcus. Others have specified that it must be able to ferment raffinose but not mannitol. Torrey and Montu (1936) showed, contrary to one claim which had been made, that the "Bargen streptococcus" is not identical with or closely related to *Streptococcus mastitidis*; on the other hand, they found it to have some properties in common with the enterococci and concluded that it is a variant of the enterococcus.

Through the kindness of Dr. Luther Thompson, who furnished ten cultures of the "Bargen streptococcus" isolated from cases of ulcerative colitis, it has been possible to study this organism in comparison with the well established types of streptococci (Stark and Sherman, 1937). These cultures were found to have very little indeed in common with the enterococci but to be, on the other hand, very closely related to, if not identical with, *Streptococcus bovis*. As previously indicated, five of these cultures had the ability to hydrolyze starch, and, on the basis of present methods of studying streptococci, would have to be considered identical with the typical form of *Streptococcus bovis*; the other five cultures were not able to hydrolyze starch but otherwise agreed entirely with the variants of this species which do not attack starch. With respect to the non-starch-hydrolyzing variety, it may be of some interest to note that a small collection of this type has been isolated from the feces of a number of normal people, thus indicating that what appears to be the typical "Bargen streptococcus" is commonly found in the human intestine.

### *Streptococcus thermophilus*

*Streptococcus thermophilus* was discovered by Orla-Jensen (1919) who gave an excellent description of this unique organism. The typical *Streptococcus thermophilus* is a very distinct type with physiological characteristics which differentiate it clearly from any other known streptococcus. It is here included among the "viridans" streptococci since in its basic characteristics it is most closely related to that group, but the properties of the or-

ganism are so distinctive that it could well be considered as belonging to an independent division of the streptococci.

Although no known streptococcus is truly thermophilic, *Streptococcus thermophilus* grows very actively at 50°C. and slightly above, but growth does not take place at 53°C. It also has a high thermal death point—apparently slightly higher than that of the other more heat-resistant streptococci—which allows it to survive, in large numbers, heat treatments of 60 to 65°C., such as in the pasteurization of milk. On the other hand, it has a high minimum temperature of growth—about 20°C.—and is extremely fastidious in its nutritive requirements. It usually has no detectable action on red blood cells, being completely “indifferent” in blood agar cultures. *Streptococcus thermophilus* does not grow in simple solid or liquid media containing only beef extract and peptone, but demands a richer medium or one supplemented with appropriate carbohydrate material. Even on the most favorable solid medium, *Streptococcus thermophilus* produces only very small “pin-point” colonies, and a general characteristic of the organism is its lack of viability in artificial media, causing it to be easily lost in laboratory cultures.

As may be noted from table 3, *Streptococcus thermophilus* is marked more by the things which it cannot do than it is by its positive reactions. It is one streptococcus which could be almost positively identified on the basis of its fermentation reactions alone. Its almost invariable inability to ferment maltose is unique. In our own work covering many cultures, in addition to those which have been published (Sherman and Stark, 1931), we have not had a strain of this organism which fermented maltose, but Orla-Jensen (1919) and others have noted a slight fermentation of this sugar by some strains. *Streptococcus thermophilus* seldom ferments raffinose and has never been reported to ferment inulin, glycerol, mannitol, sorbitol or salicin. Orla-Jensen (1919) noted that *Streptococcus thermophilus* is especially favored by sucrose and produces a strong fermentation of this substance; at the same time he noted that the monosaccharide mannose was only weakly fermented. Wright

(1936a, b) has confirmed and extended these observations. He has shown that certain strains of *Streptococcus thermophilus* utilize lactose and sucrose more readily than the constituent monosaccharides, as indicated by a faster rate of fermentation. On the basis of these results, Wright believes that *Streptococcus thermophilus* ferments the disaccharides lactose and sucrose without preliminary hydrolysis. Among the other unusual characteristics of *Streptococcus thermophilus* is its extreme sensitivity to sodium chloride, it being inhibited by only 2 per cent of this salt. No other known streptococcus is so sensitive to salt; and in this respect, as well as in certain other characteristics, *Streptococcus thermophilus* shows a physiological kinship to *Lactobacillus bulgaricus*.

*Streptococcus thermophilus* is in general characterized by the production of fairly long chains in liquid media, but in this respect it shows the variability which is characteristic of practically all streptococci. According to Orla-Jensen, this organism forms longer chains at its optimum temperature of around 45°C. than at lower temperatures, in this respect differing from the behavior of some streptococci.

The habitat of *Streptococcus thermophilus* is not known. It has never been obtained from clinical sources, and from its physiological nature it is doubtful if this organism could even rarely be implicated as a secondary invader of the tissues. Although its temperature requirements for growth might suggest that it is an intestinal organism, there is no proof that this is its source. *Streptococcus thermophilus* has been isolated only from milk and milk products. It can sometimes be isolated from quantitative plates made directly from pasteurized milk. It can also usually be isolated from milk which is incubated at 50°C. and plated soon after curdling, before the lactobacilli have gained ascendancy. *Streptococcus thermophilus* can frequently be isolated from Swiss cheese only a day or two old, since in the manufacture of this cheese a "cooking" temperature between 50 and 55°C. is employed, and the freshly made curd cools slowly and remains above 40°C. for about 15 hours, thus providing ideal temperature conditions for the growth of the organism. In

this connection, *Streptococcus thermophilus* has acquired a technical importance in that it is now sometimes used as a "starter" for the inoculation of milk in the manufacture of Swiss cheese (Frazier, 1933; Frazier, Burkey, Matheson and Watson, 1933).

#### THE LACTIC STREPTOCOCCI

Since all streptococci produce lactic acid as the chief product of fermentation, it is misleading to designate any particular group as "lactic" organisms. However, the ordinary milk-souring organism has so long been known as the "lactic-acid streptococcus" that the term has acquired a familiar technical meaning. The lactic streptococci form a homogeneous and quite distinct group. They are characterized by having low minimum and maximum temperatures of growth, strong reducing action, and a marked tolerance to methylene blue (Sherman and Albus, 1918). These features, supplemented by the other physiological characteristics of the organisms, mark rather clearly the boundaries of the group.

From the pyogenic and the viridans divisions of the streptococci, the lactic organisms are markedly differentiated by their ability to grow at 10°C., their strong reducing action, and their ability to grow in the presence of a relatively concentrated (0.1 per cent) solution of methylene blue in milk-cultures. From the enterococci, on the other hand, the lactic streptococci are differentiated by their inability to grow at 45°C., their inability to grow in the presence of 6.5 per cent sodium chloride, the inhibition of their growth in an alkaline medium with a pH of 9.6, and a somewhat lower thermal resistance (Sherman and Stark, 1934; Sherman, Stark and Yawger, 1937).

The lactic streptococci have acquired commercial importance through their use as "starters" in the dairy industry: They are used for the "ripening" of cream for buttermaking; for the inoculation of milk for cheesemaking, in order to produce the initial lactic acid fermentation in the curd; and for the production of artificial buttermilk and certain other types of fermented milk drinks. These useful functions, together with the rôle they play in the natural souring of milk with the suppression of putrefactive and other obnoxious bacteria, have caused

the lactic streptococci to be looked upon as desirable organisms and friends rather than foes of mankind.

Although some diversity among different types of lactic streptococci is indicated on the basis of fermentation reactions and certain other characteristics, most workers have considered these only variants from type, or at most varieties, rather than species. (Ayers, Johnson and Mudge, 1924; Hammer and Baker, 1926; Stark and Sherman, 1935; Yawger and Sherman, 1937a.) On the other hand, there have been suggestions, notably by Orla-Jensen and Hansen (1932), that this group of streptococci should be divided into a number of species based upon fermentation

TABLE 4  
*The lactic streptococci: additional characteristics*

SPECIES	AMMONIA PRODUCED FROM PEPTONE		GROWTH AT 40°C.		GROWTH IN PRESENCE OF		SODIUM HIPPURATE HYDROLYZED	STARCH HYDROLYZED	ESCULIN SPLIT	GELATIN LIQUEFIED	MILK CURDLED	FINAL pH IN GLUCOSE BROTH	ACID PRODUCED FROM											
	4 per cent NaCl	pH 9.2	0.3 per cent methylene blue in milk	+	-	Arabinose							Xylose	Maltose	Sucrose	Lactose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin		
<i>S. lactis</i> ....	+	+	+	+	+	+	-	-	+	-	+	4.5-4.0	+	+	+	+	+	*	-	-	+	-	+	
<i>S. cremoris</i> ..	-	-	-	-	+	-	-	-	+	-	+	4.6-4.0	-	-	+	+	+	*	-	-	*	-	+	

\* See table 1.

reactions. Ignoring such minor distinctions, there nevertheless appear to be two fairly clearly defined species in the group, *Streptococcus lactis* and *Streptococcus cremoris*. The physiological characteristics of these organisms, other than those given in table 1, are included in table 4.

The data given in table 4 bring out rather clearly the physiological differences between *Streptococcus lactis* and *Streptococcus cremoris*; other facts supporting this differentiation will be given in the discussion of the individual species.

### *Streptococcus lactis*

*Streptococcus lactis* was the first streptococcus described, being studied by Lister (1873, 1878) who isolated it from milk and



named it *Bacterium lactis*. There is nothing surprising in the fact that Lister gave this organism the generic name *Bacterium*; it should be remembered that *Streptococcus* as a generic name had not yet come into use, and Lister observed differences between his organism and the commonly known micrococci of his time. As the predominating organism in sour milk and as the chief causative factor in the souring process, *Streptococcus lactis* has been known through the years under a number of names, of which *Streptococcus acidi-lactici* (Grotenfeldt), *Bacterium lactis-acidi* (Leichmann) and *Streptococcus lacticus* (Kruse) had the widest usage. Following the lead of Löhnis (1909) bacteriologists have almost universally adopted the specific name applied by Lister, so that the organism is now generally known as *Streptococcus lactis*.

Although *Streptococcus lactis* has long been known and extensively studied, its exact identity was not clarified until comparatively recently. On the other hand, because of its rapidity of growth in milk with its resulting tendency to predominate in the natural souring of milk, there can be little doubt that most of the older work done on this organism really dealt with the true *Streptococcus lactis*. The complete reduction of litmus before curdling in milk cultures was looked upon as an especially characteristic property of the organism (Hastings, 1911), but this characteristic was even then known not to be peculiar to *Streptococcus lactis*. Andrewes and Horder (1906) had shown the strong reducing action of *Streptococcus fecalis* on neutral red, and MacCallum and Hastings (1899) had shown the ability to reduce litmus in milk cultures before curdling to be an especially characteristic feature of the organism which now goes under the name of *Streptococcus zymogenes*. Sherman and Albus (1918) showed that, in addition to other characteristics, *Streptococcus lactis* has a minimum growth temperature below 10°C. and a maximum temperature for growth at about 43°C., and that these properties are correlated with the characteristic action in litmus milk. The strong reducing action of the organism was also established with indigo carmine and neutral red; its tolerance to methylene blue was noted, as were also the fermentation reac-

tions. Orla-Jensen (1919) emphasized especially the fermentation reactions and recorded the action of *Streptococcus lactis* on a number of additional substances. Ayers, Johnson and Mudge (1924) considered the three most important differential characteristics of *Streptococcus lactis* to be its strong reducing action on litmus and Janus green, its ability to grow at 10°C., and its ability to produce a low final pH in media of low surface tension. These investigators were perhaps the first to study thoroughly the action of *Streptococcus lactis* on blood. They showed that the colonies of this organism on blood agar plates, while frequently of the gamma type, might also show various degrees of coloration through a weak alpha to a more typical alpha reaction, and these results have been amply confirmed by others.

*Streptococcus lactis* is generally referred to as being characteristically a diplococcus rather than a typical chain-forming streptococcus, but this varies greatly with different strains and with the conditions of growth. Some strains of this organism produce good chains in ordinary media. Heinemann (1906) showed that *Streptococcus lactis* produces chains in liquid media containing blood serum, and Sherman and Albus (1918) found the same to be true in a bile medium.

In fermentation reactions there is considerable diversity among different strains of *Streptococcus lactis*, among the substances which may or may not be fermented being arabinose, xylose, sucrose, mannitol and salicin. Orla-Jensen and Hansen (1932) have reported a strain which ferments raffinose, and Yawger and Sherman (1937a) have encountered a few cultures which do not ferment lactose. Starch is not hydrolyzed, when tested by the plate method, though a few strains have been noted which have a very slight action on this substance. That at least a portion of the diversity observed in the fermentation tests with the lactic-acid streptococci is to be ascribed to variation, rather than indicating distinct varieties, appears to be established (Sherman and Hussong, 1937).

There should be little need at this late date to discuss the old question of the relationship of *Streptococcus lactis* to *Streptococcus*

*fecalis*, but since these organisms have been confused in recent papers (Kleckner, 1935; Chapman, 1936), the subject will be briefly discussed. Until recent years there were no methods at hand for the differentiation of these two organisms, and in some of the older and better known classifications of the group *Streptococcus lactis* was not recognized as an independent species. Ayers and Johnson (1924) showed that *Streptococcus lactis* and *Streptococcus fecalis* were similar in having strong reducing action, and low minimum temperatures of growth, and in being able to produce low final pH values in media of reduced surface tension, as well as showing considerable similarity in morphological and general cultural characteristics. The work of these distinguished investigators naturally gave great weight to the view that *Streptococcus lactis* and *Streptococcus fecalis* were identical. Since that time, however, methods have been applied to the study of the streptococci which appear to differentiate very clearly these two species. (Sherman and Stark, 1931, 1934; Sherman, Mauer and Stark, 1937.) As was shown in table 1, *Streptococcus fecalis* has a higher maximum temperature of growth and is more tolerant to salt and alkali than is *Streptococcus lactis*. In addition, *Streptococcus fecalis* has a slightly higher thermal death point than does *Streptococcus lactis*. Even on the fermentation tests the two species appear to be fairly distinct: *Streptococcus lactis* does not ferment glycerol or sorbitol and may or may not ferment mannitol; *Streptococcus fecalis*, on the other hand, usually ferments both mannitol and sorbitol, and frequently ferments glycerol.

The true *Streptococcus lactis* is not known to occur in natural infections of man or animals. However, Heinemann (1907) reported the building up of virulence in cultures of *Streptococcus lactis* by passage through rabbits. Hammer (1928), on the other hand, reports experiments in which the findings of Heinemann were not confirmed. Various cultures of *Streptococcus lactis* isolated from milk did not prove to be harmful to rabbits or guinea pigs. When injected intravenously into rabbits, the organisms could be recovered from the spleen and liver for a number of hours after inoculation, but repeated passage through rabbits failed to yield cultures capable of causing death.

The habitat of *Streptococcus lactis* has been somewhat of a mystery: It has long been established that this organism is not a normal inhabitant of the bovine udder (Rogers and Dahlberg, 1914; Evans, 1916; Ayers and Mudge, 1922), and although older reports had indicated that the source of *Streptococcus lactis* might be the mouths and intestines of cows, Ayers and Mudge (1923), with more modern methods of study, did not find this organism among the characteristic streptococci of the bovine mouth, throat, or feces. Stark and Sherman (1935) isolated *Streptococcus lactis* repeatedly from certain plants, but not from all plants examined. On the basis of these findings it was suggested that plants may represent the natural habitat of *Streptococcus lactis*, though definite conclusions to this effect were not drawn. In view of the fact that *Streptococcus lactis* does commonly occur on plant materials, it would seem likely that surviving strains would sometimes be found in the feces of animals.

#### *Streptococcus cremoris*

There has long been a belief among some of those engaged in the scientific aspects of the dairy industry that the best lactic-acid streptococci for "starter" use have more tendency to form chains and to produce a slightly viscous body in milk cultures than does the typical *Streptococcus lactis* obtained from spontaneously soured milk. Such an ill-defined type has for years been known as a variety of *Streptococcus lactis* and also under the specific name of *Streptococcus hollandicus* (Weigmann). This organism was given more standing as an independent type by the work of Orla-Jensen (1919) who described it as a new species under the name of *Streptococcus cremoris*. He showed that *Streptococcus cremoris* is usually a more typical chain-forming streptococcus than is *Streptococcus lactis*; that it frequently fails to grow at 37°C.; usually produces less acid in milk; and in general has less fermentative power than does *Streptococcus lactis*, especially on maltose and dextrin. Although the work of Orla-Jensen indicates the validity of *Streptococcus cremoris* as a separate species, the differences between this organism and *Streptococcus lactis*, as defined by him, are relative or quantitative ones rather than definitive.

Quite independent of the work of Orla-Jensen, and uncorrelated with it, is that of Ayers, Johnson and Mudge (1924) who described a unique type of lactic-acid streptococcus as *Streptococcus lactis* Var. B. The outstanding feature of this "B" variety was its inability to produce ammonia from peptone, while the typical *Streptococcus lactis* is able to produce this substance in 4 per cent peptone solutions. Other less sharply defined characteristics of *Streptococcus lactis* Var. B were a higher average limiting pH in glucose broth and a less vigorous action in milk cultures than that of the typical *Streptococcus lactis*.

Suspecting that the organisms described by Orla-Jensen and by Ayers, Johnson and Mudge were the same, Yawger and Sherman (1937b) followed the lead of the latter and from commercial starters and milk isolated cultures of lactic streptococci which did not have the ability to produce ammonia from peptone. By the application of some new tests, it was found possible to correlate with the inability to produce ammonia other characteristics which differentiated this type from the typical *Streptococcus lactis*. This type in turn also had the characteristics of Orla-Jensen's *Streptococcus cremoris*. As may be seen from table 4, *Streptococcus cremoris* is not only unable to produce ammonia from peptone, but is also unable to grow at 40°C., in the presence of 4 per cent sodium chloride, or in an alkaline medium of pH 9.2; *Streptococcus lactis*, on the other hand, is able to produce ammonia from peptone, and is not inhibited in its growth by a temperature of 40°C., by 4 per cent sodium chloride, nor at a pH of 9.2. *Streptococcus lactis* also shows a greater average tolerance to methylene blue and in general has greater fermentative power than does *Streptococcus cremoris*, but a clear differentiation apparently cannot be based on the fermentation tests.

With regard to morphology, *Streptococcus cremoris* is, as a rule, more typically chain-forming than is *Streptococcus lactis*; however, some strains occur more typically as diplococci, while some strains of *Streptococcus lactis* produce chains, thus preventing a clear-cut distinction on this basis. In many cases the cells of

*Streptococcus cremoris* are distinctly larger than those of *Streptococcus lactis*, but again there are so many exceptions to the rule that this characteristic cannot be relied upon. It may be said, however, that lactic streptococci which are characterized both by large cells and by the formation of long chains in milk cultures offer presumptive evidence of being *Streptococcus cremoris* rather than *Streptococcus lactis*.

Generally speaking, *Streptococcus cremoris* does not grow so well in artificial media as does *Streptococcus lactis*, and it is also slightly less acid-tolerant, as revealed by a lower average production of acidity in milk and by usually not reaching quite so low final pH values in glucose broth.

*Streptococcus cremoris* has been isolated only from milk and milk products. As this organism not only cannot grow at 40°C. but a majority of strains do not grow even at 37°C., it seems unlikely that *Streptococcus cremoris* occurs as an animal parasite and scarcely possible that it could ever be implicated as an agent of disease.

#### THE ENTEROCOCCI

Since its use by Thiercelin (1899a, 1899b, 1902) the term "enterococcus" has had a somewhat variable and hazy meaning. In some cases the name has been applied rather specifically as a synonym for *Streptococcus fecalis*, but most workers have used "enterococcus" in a loose group sense to designate the fecal streptococci which have in common some of the outstanding characteristics of *Streptococcus fecalis*. A fecal diplococcus or short-chained streptococcus somewhat resistant to heat, with the ability to ferment mannitol and a tolerance for bile, would fully meet the requirements of most investigators for classification as an enterococcus; while some workers have so classified their organisms without the use of all of these characteristics. It should be noted that although the foregoing features are characteristic of the enterococci, not one of these properties is limited to that group of the streptococci. Through the application of a wider assortment of tests, and from more extensive

studies of the individual species, there has gradually evolved a rather definite and clearly defined enterococcus division of the streptococci.

Although there is a large body of literature dealing with the enterococci, only a few of these papers record the characteristics of the organisms dealt with in sufficient detail to make it profitable to review them. The older literature was reviewed by Dible (1921) and the more recent literature has been reviewed by a number of workers. (Bagger, 1925, 1926; Dible, 1929; Deme-

TABLE 5  
*The enterococci: additional characteristics*

SPECIES	LANCEFIELD GROUP	HEMOLYSIS	GELATIN LIQUEFACTION	STRONG REDUCTION	ACTIVELY FIBRINOLYTIC	SODIUM HIPPURATE HYDRO- LYZED	STARCH HYDROLYZED	ESCULIN SPLIT	MILK CURDLED	FINAL pH IN GLUCOSE BROTH	ACID PRODUCED FROM											
											Arabinose	Maltose	Sucrose	Lactose	Trehalose	Raffinose	Inulin	Glycerol	Mannitol	Sorbitol	Salicin	
<i>S. fecalis</i> ..		-	-	+	*	-	±	-	+	+	4.5-4.0	±	+	±	+	+	±	-	*	±	+	+
<i>S. liquefa- ciens</i> ....		-	+	+	*	-	±	-	+	+	4.5-4.0	±	+	+	*	+	±	-	+	+	+	+
<i>S. zymo- genes</i> ....	D	+	±	+	*	-	±	-	+	+	4.5-4.0	±	+	+	*	+	±	-	+	*	+	+
<i>S. durans</i> ..		+	-	-	-	±	±	-	+	+	4.5-4.0	-	+	-	*	+	±	-	-	*	+	±

\* See table 1.

ter, 1929.) Dible's contribution was an excellent one, and among the other worthwhile contributions should be mentioned the work of Bagger (1926) who used a wide assortment of test substances and also made significant observations on the temperature and pH range of growth of the enterococci. In general, however, it may be fairly said that most of the more significant contributions to our knowledge of the nature of the enterococci have been made by those workers who have studied individual species, rather than dealing with the "enterococcus" in the loose and hazy sense in which this term has more generally been used.

On the other hand, it should be admitted that some of the

“species” of the enterococcus division of the streptococci are separated from each other by rather thin and shaky boundaries, as may be seen from an inspection of table 5.

The enterococci present many points of interest: Not only are they characterized by unique physiological properties, as was shown in table 1, but in this group of streptococci there is a merging of hemolytic and non-hemolytic strains in what appears to be an otherwise physiologically homogeneous type; and in this group, which contains the only known proteolytic streptococci, there is likewise a fusion of proteolytic and non-proteolytic strains in the same otherwise homogeneous types, for which only convenience justifies designation as separate species. As was previously mentioned, motility in the streptococci has been especially allied with members of the enterococcus group.

Although the enterococci are considered as having their origin in the intestines of man and other warm-blooded animals, the resistance and tolerance of these streptococci, together with their low minimum and high maximum temperature limits of growth, not only fit them to survive but also to grow under diverse conditions in nature. The so-called *Streptococcus apis* (Maassen) which is associated, probably only as a secondary invader, with European “foulbrood” of bees appears to be an enterococcus. Thompson and Thompson (1928) claimed the identity of *Streptococcus apis*, *Streptococcus zymogenes* and *Streptococcus liquefaciens*; Hucker (1932) found the cultures of *Streptococcus apis* studied by him to be similar to *Streptococcus liquefaciens*; while Davis and Tarr (1936), who studied both proteolytic and non-proteolytic types, identified them with *Streptococcus liquefaciens* and *Streptococcus fecalis* (“*Streptococcus glycerinaceus*”). The enterococci grow well in milk and certain milk products, notably cheese. This fact has been partially responsible for the widespread confusion of *Streptococcus fecalis* and *Streptococcus lactis*; while certain of the hemolytic members of the enterococcus group have sometimes been designated as “dairy types.” Unpublished investigations have shown enterococci of the *Streptococcus fecalis* and *Streptococcus liquefaciens* types to occur rather commonly on



plants. This may of course mean that these organisms were merely surviving, rather than growing, under these conditions. An interesting point in this connection, and one which might be considered as evidence against such an accidental occurrence is the fact that none of the hemolytic types of the enterococci have thus far been isolated from plant materials; but no systematic investigation has been made with the specific object of obtaining these types of hemolytic streptococci from vegetable sources.

### *Streptococcus fecalis*

*Streptococcus fecalis* was named by Andrewes and Horder (1906) who gave an excellent description of the organism in view of the means then at hand for studying bacteria. The organism was described as non-hemolytic, having strong reducing action on neutral red, coagulating milk and fermenting the disaccharides, salicin, and mannitol, but not inulin and usually not raffinose. The fermentation of mannitol was considered very characteristic. *Streptococcus fecalis* was found to be the predominating streptococcus in human feces and from that time it has been known as the most characteristic streptococcal type of the human intestine.

The work of Andrewes and Horder was verified by a number of subsequent workers on intestinal streptococci. (Winslow and Palmer, 1910; Fuller and Armstrong, 1913; Broadhurst, 1915; and others.) In later classifications the identity of *Streptococcus fecalis* became less distinct, as bacteriologists came to depend more on a smaller number of tests and attention to some of the important physiological reactions was largely discontinued. In one such classification (Gordon, 1922) *Streptococcus fecalis*, or the "*enterococcus*," was identified merely as a non-hemolytic streptococcus which fermented mannitol but not raffinose.

Dible (1921), however, subjected the intestinal streptococci to a careful study which resulted in a broader and more accurate description of *Streptococcus fecalis*. Ayers and Johnson (1924) likewise studied this organism on a broader base, taking into consideration a variety of physiological characteristics. Although

he did not integrate his work with that of other investigators, and used new terminology, Orla-Jensen (1919) nevertheless made valuable additions to the existing knowledge of the physiology of *Streptococcus fecalis*, which he described under the specific names of *Streptococcus faecium* and *Streptococcus glycerinaceus*. Although Orla-Jensen based his classification mainly on the results of fermentation tests with 18 substances, he made valuable observations on the thermal resistance and the temperature ranges of growth of the organisms studied. As previously noted, especial emphasis has been laid in recent studies on the growth tolerance of *Streptococcus fecalis* and other enterococci to relatively high and low temperatures and to a number of substances which are in general inhibitory to other streptococci.

Assuming, in deference to custom and convenience, that the closely related hemolytic and proteolytic types should be considered as distinct species, the remaining organisms which are generally classified as *Streptococcus fecalis* form a rather homogeneous physiological group. There appears to be relatively little variation from type so far as the more basic characteristics are concerned; but it is likely that deviation from the usual pattern will be found more common as experience increases and more incisive tests are developed and applied to the study of non-hemolytic streptococci. For example, strong reducing action, long known as an outstanding characteristic of *Streptococcus fecalis*, is found to be lacking in a small proportion of cultures (Sherman, Mauer and Stark, 1937) and a few strains appear to lose this property on long cultivation in the laboratory.

With respect to the fermentation tests, however, *Streptococcus fecalis* shows great diversity, a fact which has been noted by all workers who have applied a broad list of test substances to the study of this organism. Arabinose, sucrose, raffinose and glycerol may or may not be fermented. Although the fermentation of mannitol has long been looked upon as an especially constant characteristic of *Streptococcus fecalis*, a few strains fail to attack this substance, as was shown by Dible (1921), whose observation has been confirmed by others. Inulin is only rarely attacked, but a small percentage of strains are able to ferment it.

Although suggestions have been made that the group should be subdivided on the basis of the fermentation reactions—for example, the *Streptococcus glycerinaceus* of Orla-Jensen—Sherman, Mauer and Stark (1937) were unable to find correlations which appeared to justify such a procedure.

In addition to being the most abundant streptococcus in the human intestine, it is probable that *Streptococcus fecalis* occurs generally in other animals, most positively in certain ones. The fact that some animals, notably the horse and the cow, harbor other types which make up the prevailing streptococcus flora has led to statements which imply that *Streptococcus fecalis* is peculiar to the human intestinal tract; but a number of investigators have reported this organism as occurring in the feces of horses, cattle and other domestic animals.

Although *Streptococcus fecalis* is not to be classed as a pathogenic organism—scarcely more so, one would think, than *Bacterium coli*—it has been known since the time of Andrewes and Horder (1906) as occasionally occurring in cases of endocarditis and other human infections. Houston (1936) has noted many types of infections in which enterococci appeared to be implicated. It is still an open question how frequently the "*Streptococcus viridans*" of clinical workers is in fact *Streptococcus fecalis*.

### *Streptococcus liquefaciens*

The occurrence of streptococci which liquefy gelatin was known in the early days of bacteriology, and the older literature records the names of many streptococci which were supposed to have this property. One of the first such types described and the one which is frequently given priority is the *Streptococcus coli-gracilis* (Escherich). However, it is impossible now to identify any of these organisms from their recorded descriptions.

The name *Streptococcus liquefaciens* was first used by Sternberg (1892) for a streptococcus which, it must be admitted, could not now be recognized from his description. Orla-Jensen (1919) revived the name and applied it to a group of proteolytic streptococci which he studied. It is of interest to note that Orla-Jensen established this species around a type culture which von

Freudenreich (1894) had described many years before under the name of *Micrococcus casei-amari*. *Streptococcus liquefaciens* has been studied by a number of investigators since Orla-Jensen, and reference may be made to the recent work of Long and Hammer (1936) in which the literature is reviewed.

Some rather fine lines have been drawn in the establishment of species in the enterococcus group and the standing of *Streptococcus liquefaciens* in this respect may well be questioned. As is shown in table 5, the only clear-cut difference between this so-called species and *Streptococcus fecalis* is proteolytic action; while from proteolytic strains of *Streptococcus zymogenes*, on the other hand, the differentiation is based solely on hemolytic activity. There is, therefore, only a slender basis for considering *Streptococcus liquefaciens* as a species independent of its closely related forms. As a matter of fact, some workers have long looked upon such gelatin-liquefying organisms simply as proteolytic varieties of *Streptococcus fecalis*, while others have considered them to be non-hemolytic strains of *Streptococcus zymogenes*.

As to whether or not *Streptococcus liquefaciens*, like *Streptococcus fecalis*, may occasionally be the cause of human infections there is little information, since non-hemolytic streptococci from such sources are not routinely tested for gelatin-liquefying power. However, Elser and Thomas (1936) have found that the gelatin-liquefying streptococci recovered in pure culture from the blood in subacute cases of endocarditis are characteristically non-hemolytic.

The primary source of *Streptococcus liquefaciens*, as in the case of the other enterococci, is probably the intestines of man and other warm-blooded animals. It is widely distributed, however, and its hardy nature equips it for growth under diverse conditions. It is frequently found in dairy and other food products in which it is able to grow vigorously. This organism has been isolated from plants and some of the strains obtained from this source are much more actively proteolytic than are the characteristic types obtained from stools and milk. Based on what is now known, probably as good reasons as any for considering *Streptococcus liquefaciens* as a species independent of its hemolytic

relative, *Streptococcus zymogenes*, are the apparently wider natural occurrence of *Streptococcus liquefaciens*, and the stronger proteolytic action of many of its strains.

*Streptococcus zymogenes* (Lancefield Group D)

MacCallum and Hastings (1899) reported an organism obtained from a case of endocarditis which they described under the name of *Micrococcus zymogenes*. They gave a remarkably clear morphological and cultural description of this organism and pointed out its resemblance to *Streptococcus pyogenes* and the pneumococci on the one hand, and to the pyogenic staphylococci on the other. The organism was found to liquefy gelatin and to digest casein; and among the other unique characteristics to which attention was called was a strong reducing action in litmus milk, the litmus being completely reduced before coagulation of the milk. They also noted that glycerol stimulated the growth of this organism, plainly indicating its utilization. Although the hemolysis of blood was not in use as a test at the time of MacCallum and Hastings' work, their description is sufficiently clear and comprehensive to mark rather definitely the type of organism with which they dealt. At the present time, the only adequately described streptococci which fulfill entirely the combination of characteristics given by MacCallum and Hastings to their organism are the hemolytic *Streptococcus zymogenes* and the non-hemolytic type which is sometimes separately designated as *Streptococcus liquefaciens*.

MacCallum and Hastings indicated that their organism was probably a common intestinal form since types which appeared to be identical had also been isolated from sewage. Shortly after the work of MacCallum and Hastings, this organism was found in specimens from human autopsies by Harris and Longcope (1901), and since that time it has been reported occasionally from clinical and fecal sources. (Birge, 1905; Hicks, 1912; Torrey, 1926; Frobisher and Denny, 1928; Sherman and Stark, 1931; Torrey and Montu, 1934; Elser and Thomas, 1936; Sherman, Stark and Mauer, 1937.)

Although given the generic name *Micrococcus* by MacCallum

and Hastings, this organism was early recognized as belonging to the genus *Streptococcus* (Winslow and Winslow, 1908) and most of the more recent investigators have so classified it.

In connection with *Streptococcus zymogenes*, there is little need to review the relatively large body of literature which has accumulated on "hemolytic enterococci" since most of the discussions given in these publications are too indefinite to give an accurate idea of just what organisms were studied. Likewise, there are numerous papers which have dealt with hemolytic streptococci obtained from feces which do not give sufficient information to allow one to judge whether or not the organisms isolated were in fact enterococci. Doubtless many if not most of these workers dealt with *Streptococcus zymogenes* or a closely related type, but with the exception of a few, such as Weatherall and Dible (1929) and Meyer (1926), it is not safe to draw such a conclusion.

In the work of O. T. Avery and Cullen (1919) and R. C. Avery (1929a) on hydrogen-ion and methylene-blue tolerance of hemolytic streptococci, there was obtained from cheese a type which was characterized by the production of a lower final pH in glucose broth and a greater tolerance to methylene blue than were human and bovine pathogenic forms. Eight of these cultures served as the foundation for the Lancefield (1933) group D; and Lancefield and Hare (1935), who obtained a larger collection of group D streptococci from the human vagina, pointed out that these organisms are in reality more closely related to *Streptococcus fecalis* than to *Streptococcus pyogenes*, thus allying them with the enterococcus group. Hare and Maxted (1935) then found group D streptococci in feces and related these to the hemolytic enterococci of previous workers. On the basis of physiological studies, Sherman, Stark and Mauer (1937) concluded that the cultures of group D hemolytic streptococci tested by them were the same as their own cultures of *Streptococcus zymogenes*, and this conclusion has been subsequently verified by the serological classification of a fairly large collection of strains of *Streptococcus zymogenes* isolated from feces and from milk.

Although *Streptococcus zymogenes* has always been considered a proteolytic organism, Sherman, Stark and Mauer (1937) concluded that non-proteolytic strains of this organism should be recognized. This suggestion was based upon the fact that hemolytic but non-proteolytic strains appeared to be otherwise entirely identical with the proteolytic type. The soundness of this view is substantiated by the serological classification of *Streptococcus zymogenes*, proteolytic and non-proteolytic types both falling in the Lancefield group D. More recent investigations (Smith and Sherman, 1937; Niven and Sherman, 1937) with collections from human feces and from milk indicate, contrary to former impressions, that the non-proteolytic type is in fact the prevailing one. The loss of proteolytic power is well known to occur in the *Proteus* and *Aerobacter* groups, and it requires no fundamental adjustment in our ideas to recognize the occurrence of proteolytic and non-proteolytic strains in the same streptococcus species.

As has already been noted, the differentiation of the so-called *Streptococcus liquefaciens* from *Streptococcus zymogenes* may be an artificial separation. Although *Streptococcus zymogenes* has usually been designated as hemolytic, several investigators have made no distinction between hemolytic and non-hemolytic types, thus considering *Streptococcus zymogenes* as giving diverse actions on blood. Indeed, Elser and Thomas (1936), on the basis of strains obtained from clinical sources, consider the non-hemolytic type as the prevailing form of *Streptococcus zymogenes*. As in the case of *Streptococcus mastitidis*, it is entirely reasonable to consider *Streptococcus zymogenes* as a species containing both hemolytic and non-hemolytic forms. The loss of hemolytic power in laboratory cultures of other types of streptococci has been reported a number of times, and though most of these observations might be considered somewhat equivocal, the results of Lancefield (1934b), previously referred to, and those of Grinnell (1928) with single-cell cultures are quite convincing.

In view of the apparent variability in hemolytic and proteolytic properties of organisms belonging to this general type, Sherman, Stark and Mauer (1937) have suggested that *Strepto-*

*coccus zymogenes* and *Streptococcus liquefaciens* might be considered simply as varieties of *Streptococcus fecalis*, the general group becoming one species with its several varieties:

- Streptococcus fecalis* (hemolysis -, proteolysis -)
- S. fecalis* var. *hemolyticus* (hemolysis +, proteolysis -)
- S. fecalis* var. *liquefaciens* (hemolysis -, proteolysis +)
- S. fecalis* var. *zymogenes* (hemolysis +, proteolysis +)

However rational such a consolidation might be, it is probable that bacteriologists will continue to classify proteolytic, and hemolytic, streptococci of this group as separate species, and almost certainly when these two characteristics are combined in the same organism. As lines between species are now generally drawn in bacterial taxonomy, there is ample precedent as well as convenience in recognizing the proteolytic and hemolytic *Streptococcus zymogenes* as a species type distinct from the non-proteolytic and non-hemolytic *Streptococcus fecalis*. Such a differentiation leaves as connecting links, or varieties, those types which are proteolytic but not hemolytic (*Streptococcus liquefaciens*) or hemolytic but not proteolytic. If these "connecting links" are not to be considered as species, it is probably more logical to consider them as varieties of *Streptococcus zymogenes* rather than to assign them to *Streptococcus fecalis*, inasmuch as mutation with the loss of characters is more common than the mutation which results in the acquisition of new characters.

In considering the close relationship of the members of the enterococcus group to one another, a few words may be said about their serological kinship. Lancefield (1937) has shown that the extracts from a few representative strains of *Streptococcus fecalis* and *Streptococcus liquefaciens* react with her group D antisera, thus appearing to belong to group D. Following this lead, we have tested more than 20 cultures each of these two organisms against group D sera prepared by the use of *Streptococcus zymogenes* as the immunizing agent. All of these cultures gave definite reactions, most of them strong. Of some interest is the fact that a number of the strains of *Streptococcus liquefaciens* were strongly proteolytic types isolated from plants, and that these also reacted with the group D sera. Sweeping



statements concerning the serological grouping of *Streptococcus fecalis* and *Streptococcus liquefaciens* would not be justified at this juncture, but it does appear on the basis of what is now known that the Lancefield group D, like group B, contains non-hemolytic as well as hemolytic members. From the statement which occurs in the abstract of Houston's address (1936) it would appear that somewhat similar results have been obtained by European workers: "With Lancefield's technique, Graham showed all enterococci examined to belong to one group."

*Streptococcus zymogenes* is the most characteristic hemolytic streptococcus of the normal human intestine, isolations made both by direct and selective methods indicating that it is commonly if not usually the predominating hemolytic form. It has little or no virulence for laboratory animals, and in spite of its clinical history as an occasional invader of the human body, *Streptococcus zymogenes*, in common with the other enterococci, is to be considered as essentially non-pathogenic. On the other hand, the difference between statistical importance and total human importance should be kept in mind. Some investigators (Houston, 1934, 1936) are convinced that *Streptococcus zymogenes* and other enterococci should be considered as significant factors in human health.

### *Streptococcus durans*

Although not formally classified, *Streptococcus durans* has been known for more than a decade as an organism which is occasionally found in milk and milk products. This hemolytic streptococcus was first isolated from a baby food which contained powdered milk and was looked upon by the original investigators as being *Streptococcus pyogenes*, or at least a hemolytic streptococcus of probable importance from the standpoint of human health. Although cursory studies of this streptococcus showed that it was physiologically clearly different from the pathogenic types of hemolytic streptococci, and had no virulence for laboratory animals, the confusion caused by its occasional isolation from dairy products led Sherman and Wing (1935, 1937) to study this organism in detail and name it *Streptococcus durans* because of its rather marked tolerance to heat and desiccation.

On the basis of its physiological characteristics, *Streptococcus durans* was related by these workers to the enterococci, though it was not then known to be of intestinal origin. Recent studies of the hemolytic streptococci of the human intestine (Smith and Sherman, 1937) have shown that this organism commonly occurs in human feces. Although a number of species of hemolytic streptococci may be isolated from human feces, so far as present information extends *Streptococcus durans* and *Streptococcus zymogenes* are the only intestinal types which may properly be designated as "hemolytic enterococci."

As is shown by the data contained in tables 1 and 5, *Streptococcus durans*, on physiological grounds, is clearly a member of the enterococcus division of the streptococci; this relationship is made obvious by its temperature limits of growth, heat resistance, and tolerance to salt, alkali and methylene blue. In common with the other enterococci, *Streptococcus durans* is bile tolerant, and although it is strongly hemolytic on blood agar plates, its hemolysin is destroyed in broth cultures so that a negative reaction is usually obtained when it is subjected to the conventional test for the formation of "soluble hemolysin." All of the cultures studied by Sherman and Wing hydrolyzed sodium hippurate, and only one of 40 strains was able to ferment sucrose; however, the more recent study of strains obtained from a wider variety of sources has shown that sodium hippurate may or may not be attacked, as is true of the other species of the enterococci, while a slightly larger proportion of strains ferment sucrose than was indicated by the earlier work.

Although it would appear that *Streptococcus durans* should unquestionably be classified as an enterococcus, the physiological reactions indicate that it is more distinctly differentiated from either *Streptococcus fecalis* or *Streptococcus zymogenes* than are the latter two organisms from each other. From the hemolytic *Streptococcus zymogenes*, *Streptococcus durans* differs in not having such strong reducing action, in never being proteolytic, and in its inability to ferment so many of the test substances; *Streptococcus durans* does not ferment glycerol nor sorbitol and usually does not attack mannitol nor sucrose, whereas *Streptococcus zymogenes* generally ferments all four of these substances.

*Streptococcus durans* has not been serologically classified by the Lancefield method, but preliminary work indicates that it probably belongs to group D. *Streptococcus durans* extracts react with strong group D antisera, but not so well as do extracts of *Streptococcus zymogenes*. As a successful grouping serum has not yet been prepared with *Streptococcus durans* as the antigen, definite conclusions should not be drawn concerning its relationship to the established Lancefield groups. On the basis of these preliminary observations, however, it probably may be safely concluded that *Streptococcus durans* is serologically as well as physiologically closely related to *Streptococcus zymogenes*, and that its classification as a member of the enterococci is sound.

Based on present information, *Streptococcus durans* is to be considered simply as an intestinal streptococcus with resistant and tolerant characteristics which permit it, in common with other enterococci, to survive and grow in environments other than its natural habitat. Tests with laboratory animals indicate that it is non-pathogenic; it has not been reported from clinical sources and there is no reason for thinking that it has much if any significance from the standpoint of human health.

#### CONCLUDING COMMENT

One cannot conclude an attempt to bring some order out of the systematic relationships of the streptococci without a feeling of chagrin, not to say one of humiliation and futility. Of one thing we may rest assured: If the present attempt does not at once appear ridiculous, it will most certainly have that appearance twenty-five years hence. The question naturally arises whether or not such efforts as this are worthwhile, if they add to the confusion which exists rather than contributing to progress. Regardless of the merits or demerits of any one effort, there can be no doubt that, over a course of years, progress is made in the classification of bacteria. And such progress has value quite aside from its taxonomic aspects. Those who have the least interest in the classification of bacteria, in a formal sense, are still engrossed in the differentiation of closely related microorganisms, by one means or another, for the attainment of practical ends.

Many workers have struggled with the question of what constitutes a species among bacteria, or even whether such a unit can be defined. The statement of Andrewes (1906), now more than thirty years old, is still good; some of it may seem a little trite at this late date, but his idea about variability in "dominant genera" perhaps still has pertinence in connection with the streptococci.

It may very properly be asked whether the attempt to define distinct species, of a more or less permanent nature, such as we are accustomed to deal with amongst the higher plants and animals, is not altogether illusory amongst such lowly organised forms of life as the bacteria. No biologist nowadays believes in the absolute fixity of species. It is recognised that those groups of like individuals, which we agree to group together for our convenience under a common name, have arisen from pre-existing species and that in many cases transitional forms can be found. It has further been made plain that the relative fixity of specific distinctions varies widely in different groups. Those forms of life which are manifestly succeeding above their fellows in the struggle for existence and are rapidly adapting themselves to new environments, show, as a rule, less specific fixity than other forms. The bounds of the individual species are ill-defined and transitional forms are numerous. Amongst British plants the brambles are often quoted as such a group; botanists have long been puzzled to define the species and have been compelled to resort to the idea of sub-species. Hooker describes no less than 21 sub-species of the common blackberry.

Such successful and variable groups are often spoken of as "dominant genera" and they are to be found as much amongst the bacteria as higher in the vegetable scale. But there are two circumstances which here render the problem of specificity even more difficult of solution. The bacteriologist is deprived of the test of mutual fertility or sterility, so valuable in determining specific limits amongst organisms in which sexual reproduction prevails. Further, the extreme rapidity with which generation succeeds generation amongst bacteria offers to the forces of variation and natural selection a field for their operation wholly unparalleled amongst higher forms of life. The machinery for the production of new varieties is enormous in the case of organisms which can provide 20 or 30 generations in a day. . . . We might almost expect the limits of species to be more intangible amongst the bacteria than amongst higher organisms. Nevertheless, many species of bacteria exhibit characters which seem quite fixed and rigid. The anthrax

bacillus and the tetanus bacillus are quite as good "species," in the natural history sense, as any that can be found amongst flowering plants. It is only when we come to the dominant genera that difficulties arise. The "bacillus coli group" is an excellent example of such a genus. The more this group is studied the more perplexing is the maze of species and sub-species, and it is significant that the tests which now enable us to pick out the chief forms are physiological and not morphological.

Now what is true of the bacillus coli group is true of the streptococci, which also form a dominant genus. Their vast abundance in nature is evidence that they have succeeded in the struggle for existence and are still maintaining their supremacy in the field which they have adapted themselves to fill. Their varieties are even more bewildering than those of the bacillus coli group and, as Gordon has shown, they can be differentiated by their physiological powers far better than by morphological structure.

The fact is that bacterial classification is still on a statistical basis—the old mountain range, mountain peak theory of species. One would think that with the passage of time, if progress is made, the species might be visualized not as peaks in a mountain range, but more like trees in a fence row connected by the inevitable underbrush. Such progress has been made, albeit the "underbrush" still bulks large. One cannot believe in evolution without believing in variation; hence the "intermediates" will long remain with us.

In a whimsical mood and quite extemporaneously, Justice Oliver Wendell Holmes once remarked:

*Facts in isolation amount to mere gossip; facts in relation become philosophy.*

And this, perhaps, gives the key to one of the chief ailments of bacterial taxonomy. There are too many "facts in isolation" and too few "facts in relation." In the laudable effort to find a few definitive tests, there has been too much tendency to center on those which are considered good, at the same time discarding others which may be of greater value in dealing with the members of closely related species. When a test is found of value in

studying certain types of streptococci it is proper to apply it to all others—but not at the expense of other reactions which may, in spite of their apparent futility, have special functions when applied to new groups. Trehalose, for example, has outstanding value in its application to the streptococci belonging to the Lancefield group C; but with the streptococci as a whole, exclusive of group C, it is not apparent that its usefulness is greater than that of maltose or sucrose. Maltose and sucrose have little general utility in the study of streptococci and have been very generally discarded. But maltose becomes quite pertinent when one is concerned with *Streptococcus thermophilus*, and also has a limited usefulness in the lactic group. Sucrose has a distinct statistical value among the streptococci belonging to the lactic and enterococcus divisions. It is recognized in other fields of biology that characters which have great differential value within one group may be quite worthless in another; but in bacteriology we have fallen prey, to a greater extent, to the fetish of standard methods and uniform charts.

It is not intended to give the impression that practical workers should apply every known test for the identification of each streptococcus studied; but for the final clarification of the various species, use will have to be made of all present methods, and of new ones yet to be developed. From such studies will evolve, eventually, the few incisive reactions needed for the identification of each species. In the meantime, imperfect or “majority” tests have distinct value in revealing the statistical pattern. As the Winslows (1908) have stated:

If the same strains are considered statistically, that is if the *frequency* of a given character be taken into account, it is apparent that certain combinations of characters are much more common than others. Measurement of almost any character by quantitative methods shows that the bacteria examined group themselves on a simple or complex curve of frequency. The modes of this curve indicate centers of variation about which the individuals fluctuate; and these centers of variation are the real systematic units of the group. The recognition of such centers, as specific types, offers the natural and satisfactory compromise between systematic multiplicity and vague generalization.

Although we are now resigned to the conclusion that probably no one physiological reaction is quite infallible, with the employment of a large number of them the better known species of the streptococci can be identified with a very high percentage of accuracy. But barring those groups which are amenable to the Lancefield technique, no comparable accuracy can yet be attained with the few tests which are commonly used in the study of these organisms. And of great importance is the fact that when streptococci are studied by means of a broad list of reactions, individual strains which are atypical with respect to some character which is normally most constant can still be identified with a considerable degree of assurance.

One naturally wonders if the goal of a simple and positive identification of all streptococcal species will be reached with serological methods. Further brilliant achievements will doubtless issue from the immunological approach; but too much should not be expected in the near future. As further use is made of the antigenic complex of organisms, the unity of living matter, as well as its diversity, becomes important. The possibilities are both manifold and complex. To illustrate, a few examples may be cited: Bliss (1937) has shown that serological type I in the Lancefield group F and type I in group G have an identical type antigen; the type II pneumococcus and one variety of the Friedländer bacillus appear to have the same soluble specific substance (Avery, Heidelberger and Goebel, 1925); and Kendall, Heidelberger and Dawson (1937) have isolated a serologically inactive polysaccharide, from mucoid strains of *Streptococcus pyogenes*, which appears to be chemically identical with that occurring in bovine vitreous humor and human umbilical cord.

But the future will doubtless take care of itself—and most probably in a manner not now anticipated. As for this review, if it helps to bring just a few facts “in relation” the effort will be justified; but the systematization of the streptococci is still far from a “philosophy.”

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# BACTERIOLOGICAL REVIEWS

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# RECENT CHEMICAL INVESTIGATIONS OF BACTERIAL TOXINS

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The chemical investigation of bacterial toxins holds much of practical and theoretical significance not only in immunology but also in physiology and chemistry. The ultimate objectives of

these studies are to determine the mode of formation of toxins, their nature, and relationships between their chemical and biological properties. This review will indicate some of the paths by which these objectives have been approached.

True toxins such as those of diphtheria and tetanus may be distinguished from other bacterial poisons by high antigenicity and the production of well-defined physiologic effects when minute amounts are injected into susceptible animals. Although the term toxin has been used to denote any poison of unknown nature, it is used in this paper to mean an antigenic poison. It is difficult to draw the line between "exotoxins" and "endotoxins" because antigenic poisons possess all degrees of toxicity and antigenicity. There are also marked differences in the ease with which toxins are liberated from bacterial cells.

Well-known biologic properties of toxins and immunological phenomena common to toxins and non-poisonous antigens will be discussed in this review only when related to the nature or mode of action of toxins. The reaction between toxin and anti-toxin could be discussed in detail more profitably in a review of antigens and antibodies in general, because this reaction probably does not differ essentially from the reaction between any other antigen and the corresponding antibody.

Nothing at all will be said about toxins such as that from *Clostridium welchii*, the toxic substances derived from gram-negative cocci, or the fibrinolysins. In general, what chemical work has been done on these is similar to that on other toxins described in this review.

## PART I. THE PRODUCTION OF BACTERIAL TOXINS

### 1. *The use of media of simplified composition*

Progress in studies on the mode of formation and the nature of toxins will be greatly facilitated when toxins are produced on media of known composition, or, at least, on media much simpler than those at present in use.

Mueller (1922 to 1937) has worked out the cultural requirements of the diphtheria bacillus so that abundant growth of some toxigenic strains may be obtained in a medium consisting of

simple organic and inorganic substances. Pappenheimer and Johnson (1936), in extending the observations of Locke and Main (1931), Scheff and Scheff (1934), and others, have shown that definite low concentrations of iron and copper are essential to toxin production. Previous failure to produce it in Mueller's medium was due to the inhibitory effect of an excess of iron. Pappenheimer, Mueller, and Cohen (1937) have described the production of potent diphtheria toxin in a medium in which the organic constituents are certain amino acids together with pimelic acid, beta-alanine, and nicotinic acid. This medium is not synthetic in the strict sense of the word, because some of the purified constituents were obtained from natural sources and may have contained impurities which stimulate toxin production. In this connection may be mentioned the finding of Pappenheimer and Johnson (1937) that an unknown inorganic constituent of soft glass stimulates the production of toxin in simplified media.

Media consisting of amino acids and small amounts of partially purified but unknown constituents have been developed by Knight, Fildes and their co-workers (1933, 1936) for growth of the staphylococcus and for *Clostridium botulinum*. Knight (1937) has found that the accessory substances necessary for the growth of the staphylococcus are nicotinic acid and vitamin B<sub>1</sub>, or closely related substances. However, toxins have not yet been produced in significant amounts in these media. Burrows (1933), using an amino acid medium, was able to produce weak toxin from type A strains of *Cl. botulinum* but not from type B strains. Tryptophane seems to stimulate slightly the production of toxin. Tani (1934) obtained growth and production of toxin by *Cl. botulinum* in a biuret-negative medium made from a sulphuric acid hydrolysate of Witte peptone.

For purposes of subsequent purification of toxins, we may use protein-free media of simplified but unknown composition from which the toxin may be precipitated selectively. The Wadsworth-Wheeler medium (1934) contains no proteins precipitable by acid but does contain proteoses precipitable by ammonium sulphate, alcohol, or acetone. Diphtheria toxoid may be precipitated by acid in a relatively pure state from this medium, but

the toxin is damaged by acid. This medium might be useful for the production and purification of other toxins that remain undamaged by precipitation with acid. A similar medium has been used by Sommer (1937) for botulinus toxin. For the production of diphtheria toxin, Pappenheimer and Johnson (1937) have described a gelatin hydrolysate medium to which certain amino acids and accessory substances have been added. It contains no substance precipitable by ammonium sulphate, and diphtheria toxin produced in it may be considerably purified simply by salting out. Holt (1937) and McLean (1937) have obtained growth and toxin production by *Staphylococcus aureus* in a medium made with the dialysate from nutrient broth. This medium contains no nitrogenous substances insoluble in saturated ammonium sulphate.

## 2. *The possible relationship of oxidation-reduction systems to the formation of toxins*

Although direct proof is lacking, a number of observations seem to link the formation of toxins to the activity of respiratory enzymes and other substances concerned in oxidation-reduction processes. In 1931, Coulter and Stone described a complex porphyrin, apparently containing both copper and iron, which is present in the toxic filtrates from cultures of *Corynebacterium diphtheriae* and is not formed by non-toxigenic strains.<sup>1</sup> It may, however, be formed by yeast and certain other organisms. The amount of porphyrin as judged by the intensity of the absorption spectrum is parallel to the amount of toxin formed. These observations were confirmed by Wadsworth, Crowe, and Smith (1935) who also showed that the porphyrin can be adsorbed out of toxic filtrates by charcoal. Wheeler and Crowe (1936) found that removal of porphyrin from, or its addition to, cultures of *C. diphtheriae* does not affect the formation of toxin. Pappenheimer and Johnson (1937) found that the amount of toxin

<sup>1</sup> Since this article has gone to press another paper by C. B. Coulter and F. M. Stone (Proc. Soc. Exp. Biol. Med., 1938, 38, 423-425) has appeared in which evidence is presented that the pigment in diphtheria culture filtrates is a zinc coproporphyrin compound.

and the amount of porphyrin can be increased or decreased in parallel by varying the concentration of iron in the medium.

These observations and the fact that iron and copper are essential for the production of diphtheria toxin indicate that the porphyrin found in the filtrates may take part in the formation of toxin in the bacillus although it has no effect when free in the culture medium.

Urban and Eaton (1937) have shown that the porphyrins derived from *C. diphtheriae* are reversibly oxidized by oxygen in the presence of reduced cytochrome C and form a redox system with lactoflavin, but, contrary to the observations of Coulter and Stone (1931), these porphyrins are not directly oxidized by potassium ferricyanide.

Levaditi and his co-workers (1934) have called attention to another substance, with an absorption band at the ultraviolet end of the spectrum, which they claim is parallel in amount to the amount of diphtheria toxin formed and is absent from culture filtrates of non-toxicogenic strains. This is denied by Ottensouer, Krupski, and Almasy (1935).

Although iron, copper, and cysteine are essential for growth and toxin production by the diphtheria bacillus (and probably other organisms), excess of any one of these substances inhibits the formation of toxin. (Locke and Main, 1931, Pappenheimer and Johnson, 1936). Kligler, Liebowitz, and Berman (1937) report that ascorbic acid added to the culture medium in a concentration of 0.1 mg. per cc. markedly reduces the formation of diphtheria toxin. The reported inhibitive effects of cysteine and ascorbic acid may have been due to the introduction of an excess of iron as an impurity in these substances.

Burky (1933) observed that when toxigenic strains of *Staphylococcus aureus* are grown anaerobically no pigment and no hemotoxin are formed but the concentration of lethal toxin is the same as in filtrates from aerobic cultures. On the other hand, anaerobic cultivation of the streptococcus or pneumococcus does not reduce the production of hemotoxin. The production of diphtheria and Shiga dysentery toxins is inhibited by anaerobic cultivation of the organisms. McBroom (1937) has found a cor-

relation between the ability of strains of staphylococcus to reduce methylene blue and to form hemotoxin. Kodama (1936) has reported that a thermolabile substance extracted in parallel with the cytochrome pigments from muscle stimulates the production of erythrogenic toxin by the hemolytic streptococcus. These observations constitute the fragmentary evidence that oxidation-reduction processes in the bacterial cell may be intimately associated with the formation of at least some of the toxins.

### *3. Other factors affecting the formation of toxins*

Because of the use of complex media made up of substances of indeterminate composition and purity, much of the work on toxin production, although of undoubted practical value, has little significance in the present connection. For this reason an extensive review of the literature on this subject has not been undertaken.

A constituent of the medium need not necessarily act directly either by stimulating growth or toxigenicity. It may act by removing an inhibitory factor, by effecting changes in pH or reducing conditions during growth, or by protecting the formed toxin from destruction. Traces of unknown impurities in an "essential" substance may stimulate the formation of toxin. The success of Taylor (1935) in producing very strong diphtheria toxin in a hog-stomach-digest medium is due to factors as yet unknown. The work of Pappenheimer, Mueller, and Cohen (1937) and Pappenheimer and Johnson (1936, 1937) indicates that in a medium containing the organic and inorganic substances required for growth of the diphtheria bacillus, the production of toxin may be brought about by the addition of the proper amounts of inorganic substances. The latter investigators have pointed out that the amount of iron necessary to inhibit toxin formation is considerably less than that found in normal tissues. An excess of iron may also occur in peptones or sugars. Most methods for preparing media for the production of diphtheria toxin involve a step in which phosphates of metal ions are precipitated in alkaline solution. The excess of iron inhibitory to toxin production is carried down and removed in this precipitate.

The manner in which carbon dioxide and soft agar act in stimulating the formation of toxin by the staphylococcus has been the subject of considerable investigation. Bigger (1933) found that good toxin is produced by some strains in a medium containing glycerol and phosphate buffer without carbon dioxide; but McLean (1937) has had less success in substituting other buffers for carbon dioxide. This suggests that the carbonic acid may act as a buffer, or maintain the pH at the optimum level for toxin production. Carbon dioxide as such, and independently of its effect on pH, stimulates the growth of bacteria, and it may in some way affect the metabolic processes concerned in the formation of toxin.

Until recently the use of soft agar for the production of staphylococcus toxin has been considered essential. However, McLean (1937) has found that after adsorption from the medium of an inhibitory substance by kieselguhr, kaolin, filter paper, or cellophane, good toxin may be produced without agar. McLean believes that agar acts in a similar way.

Jordan and Burrows (1935) report that the production of enterotoxic substances by strains of staphylococcus concerned in food poisoning is greatly increased by adding starch to the medium and cultivating the organisms on soft agar under 20 per cent carbon dioxide. After repeated transfer to this medium certain strains of *Streptococcus viridans*, *Bacillus proteus*, and members of the colon-typhoid group also acquire the ability to produce gastro-intestinal poison. It is possible that the enterotoxic substance is a metabolite, not a true toxin, because it is non-antigenic, and, unlike other well-defined toxins, it is soluble in organic solvents (Jordan and Burrows, 1933).

#### 4. *Toxins as products of secretion, autolysis, or the action of enzymes on the medium*

The production of diphtheria toxin in a medium containing only substances of the degree of complexity of amino acids, and the production of other protein-like toxins in media containing no proteins or proteoses makes it unlikely that these toxins are formed by enzymic degradation of a constituent of the culture



medium. It seems rather that toxins are synthesized in the bacterial cell and then liberated, by diffusion into the medium or by disruption of the cell. Most toxins are intermediate in their properties between two extremes represented, on the one hand, by diphtheria toxin, which appears in the medium even during the first hours of growth and is easily washed out of the bacilli, and, on the other, by the toxic substances in the bodies of the colon-typhoid organisms which are liberated only by prolonged autolysis, tryptic digestion, or extraction with acids.

Nelson (1927) found that intact botulinus bacilli are very toxic and their toxicity is not appreciably diminished by washing. The toxin is apparently combined with a protein of the bacterial cell which can be removed by peptic digestion. More recently Sommer (1937) has reported that botulinus toxin having almost the same activity as the purified toxin separated by him from filtrates can be obtained by dissolution of the dried bacilli in phosphate buffer.

Both the neurotoxin and the enterotoxin of the Shiga dysentery bacillus exist in the bacterial bodies (Boivin and Mesrobian, 1937b). The neurotoxin is liberated from the cells by diffusion in an alkaline medium, but the enterotoxin is liberated only by autolysis. Hansen (1936) has obtained strong dysentery toxin by grinding the dried bacilli in water and concentrating the toxin by adsorption and precipitation. Gildermeister and Grillo (1935) obtained increased production of toxin by growing the cultures in broth at pH 8.8 inside a cellophane bag immersed in broth. This was attributed to the escape of metabolic products through the membrane, thus permitting better growth of the bacilli.

In 1934, Weld described a heat-labile hemotoxin of the streptococcus which, after intravenous injection, kills mice and produces extensive intravascular hemolysis. Unlike the pneumococcus hemotoxin which is generally liberated by breakdown of the cells and the staphylococcus hemotoxin which is formed as a soluble substance during growth, the streptococcus hemotoxin is best obtained by treating the organisms with serum. Several successive serum extracts contain hemotoxin of equal potency, and extraction of a larger mass of the cocci with the same amount of serum does not increase the potency. This indicates saturation

of the serum or limitation of the amount of toxin formed by the availability of something in the serum. Schluter and Schmidt (1936) have repeated the work of Weld, and have been unable to obtain hemotoxin by extraction with Tyrode solution or solutions of gelatin, gum arabic, or peptone. With solutions of serum albumin or globulin only weak toxins were obtained. Dialyzed serum failed to extract the toxin but addition of sodium chloride restored its extracting power. Dilution or concentration of the serum also diminished the extracting power. The ability of the streptococci to produce hemotoxin diminishes after 14 hours of growth. Extraction of the organisms with saline, ether, or alcohol also destroys the ability to yield hemotoxin on subsequent treatment with serum. It is possible that this hemotoxin of the streptococcus is not extracted by the serum but is formed by the action of an enzyme of the organism on some constituent of the serum.

## PART II. THE PURIFICATION AND CHEMICAL NATURE OF BACTERIAL TOXINS

### *1. Concentration, partial purification, and separation of toxins*

This section will be devoted to the applications of chemistry in the study of toxins which have been concentrated or have been separated from a mixture of toxins but have not in most cases been purified to any great degree.

Procedures such as precipitation with alcohol, acetone, or ammonium sulphate, dialysis or ultrafiltration, and evaporation at low pressure and temperature have long been used to concentrate bacterial toxins. Such methods usually do not effect a great amount of purification except where specially designed and simplified media have been used for production of the toxin. However, concentration may be useful in the study of bacterial products having poorly defined biological properties; and simple chemical procedures may sometimes be used to separate a mixture of two or toxins produced by the same organism.

In considering the destructive effects of a chemical procedure two criteria of the amount of alteration of the toxin should always be observed:

- (1) The final yield of toxin (measured in units, such as skin

test dose, minimal lethal dose, or flocculating unit) in the purified or concentrated sample should be a major fraction of the total toxin in the original material.

(2) The toxicity per gram of dry weight in the final preparation should be equal to or greater than that of the original toxin.

*a. Toxins of the hemolytic streptococcus, pneumococcus, and staphylococcus.* Scarlatinal toxin may be concentrated by fractional precipitation with ammonium sulphate or sodium chloride according to the methods used by Huntoon (1924), Dick and Boor (1935) and others. Acetone has been used as a precipitant by Wadsworth and Quigley (1931). Precipitation with neutral salt has yielded a ten- to twenty-fold purification of the toxin without much loss of toxicity. Simple acetone precipitation has effected a five- to ten-fold purification. These methods undoubtedly involve precipitation of much inactive material from the culture medium.

In 1929 Korschun, Krestownikowa, and Rjachina precipitated scarlatinal toxin with sodium chloride and then with alcohol. The alcohol precipitate was redissolved in acidified water to separate the toxin from an insoluble nucleoprotein. The resulting toxic substance is stated to be a polysaccharide containing nitrogen. A similar substance was obtained by Kodama (1936). Huntoon, and Dick and Boor reported that their scarlatinal toxins were destroyed by tryptic digestion, but Kodama used tryptic digestion as a step in the purification of his scarlatinal toxin. Recently Stock (1937), using methods similar to those of the Japanese and the Russian investigators, has obtained a toxin of considerable activity which gives 60 per cent of reducing sugars on hydrolysis. Stock showed, however, that a similar polysaccharide can be isolated from commercial peptone. Apparently, scarlatinal toxin has not yet been isolated in a degree of purity sufficient to warrant definite conclusions as to its nature.

Separation of various substances giving skin reactions and of other toxic products from hemolytic streptococci has been partially successful but much remains to be done. Hooker (1936) has called attention to the multiplicity of toxins or poisons, elaborated by the hemolytic streptococcus, which may play a

part in infections with this organism. In 1934, Hooker and Follensby demonstrated the existence of what they termed A and B toxins of the hemolytic streptococcus. The B toxin is present in the fraction precipitated by 0.60 saturated ammonium sulphate solution, while the A toxin is precipitated at 0.75 saturation. Hooker and Follensby found strains of streptococci which produce mostly A or mostly B toxin. The NY5 strain produces A and B while the Dick strain produces only A. The two toxins differ in their stability to various physical and chemical agents. The B toxin resembles a polypeptide or protein while the A toxin in many respects departs from the properties commonly attributed to a protein. It is interesting to note that the B toxin is digested by trypsin whereas the A toxin resists digestion by pepsin, pancreatin, or trypsin.

From hemolytic streptococci and their culture filtrates, Kodama (1936) has separated several substances which give skin reactions. One of these is a scarlatinal toxin which resists tryptic digestion and which Kodama believes is similar to the A toxin of Hooker. In addition, Kodama describes an alcohol-soluble, acetone-insoluble polypeptide which gives reactions of an allergic nature in certain adults, and a nucleoprotein which also gives allergic skin reactions. The importance of separating toxic from allergic factors in streptococcic filtrates is evident in view of the discussions of these factors in papers such as those by Hooker (1933), Ando, Kurauchi, and Nishimura (1930), and Cooke (1928).

Rane and Wyman (1937a) have demonstrated a definite flocculation of scarlatinal toxin and antitoxin by using toxin concentrated and partially purified by precipitation with ammonium sulphate. O'Meara (1935) and others had previously obtained flocculation by using enormous volumes of unconcentrated toxin relative to the amount of antitoxin, and by incubating for a long time. The concentrated toxin prepared by Rane and Wyman flocculates with a suitable serum in 15 minutes. Although other investigators have failed to demonstrate a relation between the flocculation value and the skin test dose of scarlatinal toxin, Rane and Wyman report relatively constant values of about

60,000 skin test doses (as determined on rabbits) per flocculating unit, using the National Institute of Health unit of antitoxin. In most details the flocculation of scarlatinal toxin closely resembles the Ramon reaction with diphtheria toxin.<sup>2</sup>

The demonstration that hemolytic streptococci and pneumococci form lethal substances has been accomplished by purifying and concentrating these substances. The concentrated streptococcus toxin prepared by Korschun and his collaborators was sometimes fatal to rabbits in doses of 0.02 gram. Rane and Wyman (1937b) report that strong toxins containing 10 to 80 flocculating units per cubic centimeter kill adult rabbits in doses of 5 cc. Young rabbits are more resistant.

In addition to the toxins of hemolytic streptococci just discussed, three streptococcus hemolysins have been described, and two of these are apparently toxic for experimental animals. As was mentioned in the preceding section, the heat-labile substances extracted from streptococci with serum (Weld, 1934) kill rabbits and mice with extensive *in vivo* hemolysis. More recently Czarnetzky, Morgan, and Mudd (1938) have prepared a heat-stable hemolysin by extraction of frozen and dried ("lyophile") streptococci with ether. A crystalline derivative of this hemolysin is also hemolytic and lethal for mice and rabbits in doses of approximately 0.16 mg. per kilogram of body weight. These hemolysins and that described by Weld have not yet been shown to be antigenic. Consequently, they must be provisionally classified as poisons rather than as true toxins. Besides these two hemolysins which are oxygen-stable, Todd (1932) has described an oxygen-labile streptococcus hemolysin which is antigenic and probably similar to pneumococcus hemolysin. This hemolysin may be related to the oxygen-labile antigen of Group A streptococci described by Czarnetzky, Mudd, Pettit, and Lackman (1938).

By concentrating culture filtrates of pneumococcus type III by ultrafiltration, Coca and his associates (1937) have obtained a

<sup>2</sup> Ramon and his collaborators (1937a) report that tetanus anatoxin, concentrated by precipitation with trichloroacetic acid, flocculates rapidly with antitoxin.

preparation which kills mice in 1 cc. doses and produces skin reactions in rabbits. Dick and Boor (1937) have obtained a similar substance by precipitation with ammonium sulphate. The fatal dose for mice is approximately 0.05 gram. These preparations also produce skin reactions and fever in human beings. The lethal substances resemble other toxins in that they are antigenic and are neutralized by the corresponding anti-toxin. The pneumococcus toxin is somewhat unusual in being type-specific. However, the large amount necessary to kill experimental animals, and the stability to heat sharply differentiate these substances from toxins such as those of diphtheria and tetanus. The relation between the symptoms produced by the toxin and those resulting from infection with the organism has not been clearly demonstrated. It is possible that insignificant amounts of these substances are produced by pneumococci and streptococci under artificial cultivation but that larger amounts are produced *in vivo* and do play a part in pneumococcus and streptococcus infections. The development of methods for producing, concentrating, and purifying these so-called toxins will lead to a better knowledge of their biological activity.

Like the streptococcus, the staphylococcus probably produces several toxins or poisonous substances which may usefully be investigated by chemical methods. The identity or separability of hemolyzing, necrotizing, and lethal toxins has not definitely been settled. Glenney and Stevens (1935) and Roy (1937) have described  $\alpha$  and  $\beta$  staphylococcus toxins which differ in their ability to hemolyze human and rabbit red cells, to produce necrosis in the skin, and to kill susceptible animals. There are corresponding anti-toxins for these  $\alpha$  and  $\beta$  toxins. The gastrointestinal toxin described by Dack and his co-workers (1931) and Dolman (1934) apparently differs both in its chemical and biological properties from the hemolytic and necrotizing toxins of the staphylococcus. This substance is apparently non-antigenic and may not be a true toxin (Jordan and Burrows, 1933).

Some progress has been made in the purification and concentration of staphylococcus toxoid. Holt (1937) prepared toxin in a dialysate medium; and the formalin-toxoid from this toxin

was then precipitated with ammonium sulphate. The resulting product was free of the nitrogenous constituents of the medium but contained a carbohydrate derived from the agar used. Ramon, Boivin, and Richou (1936) have concentrated staphylococcus toxoid by precipitation with trichloroacetic acid at pH 4.0.

*b. Toxins of the Salmonella, Proteus, colon, and dysentery groups of organisms.* Raistrick and Topley (1934) obtained from *Salmonella aertrycke*, by digestion of the acetone-extracted bacilli with trypsin and precipitation with 68 per cent alcohol, an antigenic fraction which produces somatic "O" agglutinins in the serum of immunized animals. As shown by Martin (1934) and Delafield (1934) this fraction is toxic for mice and rabbits. Herter and Rettger (1937) have also described toxic fractions obtained from *S. aertrycke*. Substances of a nature similar to that of Raistrick and Topley have been obtained by Boivin and Mesrobeanu (1937a) from members of the *Salmonella*, *Proteus*, and colon groups by extraction of the bacterial bodies with trichloroacetic acid and precipitation of the extracted material with alcohol. These toxic substances are produced by the smooth but not by the rough variants and are independent of the presence of the H antigen. They contain from 3 to 5 per cent of nitrogen, small amounts of sulfur and phosphorus and give 20 to 40 per cent of reducing sugars on hydrolysis. An ether-soluble lipid component is split off by acid hydrolysis. Tests for peptide linkages and tyrosine are positive, but the substances are not precipitated by the ordinary reagents for proteins. This carbohydrate-lipid complex constitutes about 10 per cent of the bacterial bodies, kills mice in doses of 0.1 to 1.0 mg., produces only weak antitoxin, and is stable to heat in a neutral but not in acid or alkaline solutions.

The existence of a second but weaker endotoxin is indicated by the observation of Boivin and Mesrobeanu (1937a) that, after destroying the gluco-lipid complex by heating with dilute acetic acid, residual toxicity remains. This endotoxin, which is destroyed by tryptic digestion, is apparently a polypeptide, and is found in rough variants as well as in smooth.

Extending their observations, Boivin and Mesrobeanu (1937

b, c) demonstrated the existence of endotoxins in the bodies of Shiga and Flexner dysentery bacilli. These gluco-lipid complexes produce acute gastro-intestinal symptoms in experimental animals and are similar to the substances obtained from other gram-negative bacilli. Morgan (1936) has isolated and analyzed a specific nitrogenous polysaccharide from the Shiga bacillus. Other toxic fractions from dysentery organisms have been described by Olitski, Reibowitz, and Berman (1937).

Boivin and Mesrobian (1937c) have carried out a chemical separation of the endotoxin from the exotoxin in filtrates of cultures of Shiga dysentery bacilli. The exotoxin, which is formed by both R and S variants, is precipitated by trichloroacetic acid at pH 3.5. After complete precipitation of the exotoxin, the endotoxin remains in solution and is recovered by precipitation with alcohol. In contrast to the endotoxin, the exotoxin is destroyed by tryptic digestion and by heat, has the general properties of a protein, and acts on the central nervous system but not on the gastro-intestinal tract.

This work on the Shiga dysentery toxin has been confirmed by Haas (1937a) who separated a heat-labile neurotropic exotoxin from a heat-stable endotoxin using the methods of Raistrick and Topley and of Boivin and Mesrobian. The endotoxin prepared by Haas is biuret-negative, gives a strongly positive Molisch test, and contains only 2.25 percent of nitrogen. It is slightly more active and may, therefore, be purer than the substances obtained by other investigators. Haas points out that since nutrient broth gives a protein precipitate with trichloroacetic acid, the protein nature of the exotoxin has not been conclusively demonstrated.

## *2. Purification of bacterial toxins*

The isolation of bacterial toxins in a pure state must depend upon the simplification of culture media and the perfection of chemical methods for separating the toxins from the constituents of the culture media and from proteins and other non-toxic products of the bacteria.

Although the plant toxin, ricin, was isolated as a protein by



Osborne, Mendel, and Harris in 1905 by fractionation with ammonium sulphate, the technical difficulties of separating bacterial toxins in an unaltered condition from the mixture of proteins, proteoses, and peptones with which they are associated in crude filtrates have not been so easily overcome. The simplest and generally most successful method has been precipitation of the toxin-containing protein fraction with acid, as applied to diphtheria toxin by Watson and Wallace (1924) and Locke and Main (1928), to botulinus and tetanus toxins by Snipe and Sommer (1928) and Sommer (1937), and to diphtheria, tetanus, and staphylococcus toxins and anatoxins by Boivin and Izard (1937). It is evident, however, from recent work that diphtheria toxin and possibly other toxins are damaged by precipitation with acid. Diphtheria toxin may be purified without alteration by adsorption on aluminum hydroxide and elution with phosphate buffer (Lindstrøm-Lang and Schmidt, 1930, and others), but some proteins, proteoses, and other constituents of the culture medium are adsorbed and eluted under the same conditions as the toxin. More extensive references to the literature on the purification of diphtheria toxin will be found in the paper by Eaton (1936a).

Using a new method which consists in precipitating the toxic fraction with the salts of aluminum and cadmium under carefully controlled conditions, Eaton (1936a) succeeded in obtaining highly purified, unaltered diphtheria toxin. It was produced in the proteose peptone medium of Wadsworth and Wheeler (1934). Pappenheimer (1937) obtained one of similar purity by simple ammonium sulphate fractionation of toxin produced in a medium made from hydrolyzed gelatin, amino acids, and accessory substances from liver extract.

Partial separation of toxin from bacterial proteins has been accomplished by fractionation with ammonium sulphate and fractional adsorption of the bacterial proteins on the colloidal hydroxides of magnesium or aluminum (Eaton, 1936a, Pappenheimer, 1937). One of the bacterial proteins is precipitated by 0.33 saturated ammonium sulphate solution at pH 7.0, and it is thus easily separated from the toxin which is precipitated be-

tween 0.4 and 0.6 saturation. A second bacterial protein precipitated at the same concentration of ammonium sulphate as the toxin may be separated by precipitating the toxin at pH 5.4 in a 0.33 saturated ammonium sulphate solution (Eaton, 1937b). Although the toxin is slightly damaged by this procedure it is the only successful method yet devised. The character of the

TABLE 1  
Diphtheria toxin and anatoxin

INVESTIGATOR	M.L.D. OF BODY WEIGHT (GUINEA PIG)*	NITROGEN PER Lf UNIT*
	<i>grams per kgm.</i>	<i>mgm.</i>
Locke and Main (1928).....	Not given	0.0006-0.0008
Eaton (1936a).....	$4.0 \times 10^{-7}$	0.00046-0.00055
Pappenheimer (1937).....	$4.0 \times 10^{-7}$	0.00046
Theorell and Norlin (1937).....	Anatoxin	0.00088
Boivin and Izard (1937).....	Anatoxin	0.00045

Other toxins

INVESTIGATOR	TOXIN	TEST ANIMAL	M.L.D. OF BODY WEIGHT*
			<i>grams per kgm.</i>
Osborne (1905).....	Ricin†	Rabbit	$5.0 \times 10^{-7}$
Sommer (1937).....	Botulinus	Mouse	$2.0 \times 10^{-7}$
Sommer (1937).....	Tetanus	Mouse	$1.0 \times 10^{-6}$
Eaton (unpublished).....	Tetanus	Mouse	$4.0 \times 10^{-7}$
Eaton (1936c).....	Tetanus	Guinea pig	$1.5 \times 10^{-7}$

\* Calculated in some cases from figures given in other terms by the authors in order to make the results comparable.

† Not a bacterial toxin. Cited for comparison.

bacterial protein separated by differential adsorption on metallic hydroxides is not known at present.

Bacterial proteins in purified diphtheria toxin have been detected by precipitin tests with anti-sera against these proteins. Eaton used anti-serum prepared by injecting rabbits with washed whole diphtheria bacilli. Pappenheimer prepared anti-serum against the bacterial protein precipitated by one-third saturated ammonium sulphate; however, it seems likely that this anti-serum might fail to detect the bacterial protein which is pre-

cipitated at the same concentration of ammonium sulphate as the toxin (at 0.4 to 0.6 saturation). Estimation by the precipitin test of the amount of bacterial protein in purified toxin was made by titrating the antibacterial serum first against known amounts of bacterial protein free of toxin, and then against the preparation of purified toxin (Eaton, 1937b). The results indicate that in preparations containing the minimal amount of total protein per Lf unit, 10 to 15 per cent of the protein is bacterial precipitinogen and the rest toxin. The amount of bacterial protein may be reduced to 1 or 2 per cent by fractionation with acid as previously described, but not by simple fractionation with ammonium sulphate.

A summary of the more successful results of purification is presented in table 1. In all of these experiments the bacterial toxins have been separated from over 99 per cent of the nitrogenous impurities.

In two cases botulinus and tetanus toxins more active than the purest diphtheria toxin have been prepared. This is not surprising in view of the fact that these two toxins in the crude state are more active than diphtheria toxin. Diphtheria toxin and ricin are the only two toxins for which convincing evidence of purity has been advanced. Both are apparently proteins coagulable by heat.

### *3. Criteria of Purity*

Since it has not been possible as yet to crystallize toxins, we must use criteria of purity which are not generally applied in organic or inorganic chemistry but which are of considerable value when applied to proteins with well-defined biological properties.

The attainment of a constant ratio of weight or nitrogen content to biological activity has been applied as a criterion of purity to diphtheria toxin and ricin. Karrer and his associates (1924) were unsuccessful in an attempt to separate from ricin, prepared by the method of Osborne, Mendel, and Harris, more active fractions by methods of adsorption or precipitation. Fractions showing less activity were sometimes obtained but these were probably

toxin altered by the chemical treatment. The nitrogen per Lf unit of diphtheria toxin has not been reduced below 0.00045 milligram by repeated precipitation with a variety of reagents, by fractional adsorption on metallic hydroxides or kaolin, or by repeated fractionation with ammonium sulphate (Eaton, 1936a, 1937b, Pappenheimer, 1937). It is possible that repeated chemical fractionation of a toxin may cause an amount of destruction that equals or slightly exceeds the further purification attained. In the separation of diphtheria toxin from bacterial protein by fractionation with acid, it can be demonstrated by the precipitin test that impurities have been removed but the nitrogen per Lf is not reduced and may be, in certain instances, slightly increased because of partial destruction of the flocculating properties (Eaton, 1937b).

Measurements of physical and chemical properties such as optical rotation, molecular weight, precipitability by protein reagents, iso-electric point, and content of nitrogen and various amino acids have been used to characterize diphtheria toxin and ricin as proteins. The demonstration of the absence of impurities such as pigments, carbohydrate, proteose, peptone, and compounds containing phosphorus, from purified preparations of toxic protein constitutes evidence that the protein is the toxin. However, none of these tests proves that it is not mixed with another, atoxic, protein having otherwise similar properties.

Pappenheimer and Robinson (1937) have shown that the nitrogen per Lf unit of diphtheria toxin specifically precipitated by antitoxin corresponds to the nitrogen per Lf unit of the purest preparations obtained by chemical methods. Unless it proves possible to split off part of the flocculating protein without affecting the toxic properties, this is evidence that the purified preparations are almost pure toxin. Under the conditions of the experiment, bacterial protein detectable by anti-bacterial serum remained in the supernatant. The identity with the purified toxic protein of the substance precipitated by antitoxin from the crude preparation was not proved except by the close correspondence of nitrogen per Lf unit. Full proof would require that the experiment be performed with purified antitoxin.

Theorell and Norlin (1937) purified diphtheria anatoxin by cataphoresis in the Theorell apparatus and obtained a preparation containing about one and a half times as much nitrogen per Lf unit as diphtheria toxin purified by other methods. The authors conclude that their preparation is a pure protein because it travels as a unit in the electric field, but purer anatoxin has been prepared by purification of crude anatoxin and by detoxification of purified toxin with formaldehyde. Uniformity of motion in an electric field is not, therefore, a valid criterion of purity in this case.

Titration of the equivalent weight of ricin done by Karrer and his associates (1924) indicated that their preparation consisted of higher and lower molecular fractions although it was not possible to separate these by any of the methods used. Even crystallization does not always guarantee the uniformity of a protein as shown by Hewitt (1936) for serum albumin. Thus far, apparently no studies of purified toxins or attempts to purify these substances by the ultracentrifugation method have yet been made. Pappenheimer (1937) gives the minimum molecular weight of diphtheria toxin as about 18,000.

#### *4. Hydrolysis, digestion, and analysis of toxins*

Up to the present time, chemical analyses of purified ricin and diphtheria toxin have yielded no clue as to the nature of the toxic structures. Karrer was unable to find any unusual grouping in ricin by analyzing the products resulting from tryptic digestion and hydrolysis with acid, although two kilograms of the purified protein were available for this study. Ricin contains cysteine. Eaton (1936a) found that diphtheria toxin gives negative nitroprusside and lead acetate tests for cysteine sulfur. Pappenheimer (1937) finds that his preparations of diphtheria toxin contain 0.75 per cent sulfur but give a negative nitroprusside reaction. It is probable, therefore, that the sulfur does not exist in the usual sulfhydryl configuration. Neither diphtheria toxin nor ricin contains phosphorus; they both contain about 16 per cent nitrogen. The high content of arginine and glutamic acid in ricin indicates that this protein has many free basic and acidic groups.

Diphtheria toxin apparently also contains much arginine as indicated by the strong Sakaguchi test. Tryptophane and tyrosine are present in both diphtheria toxin and ricin. Eaton (1936a) obtained weak qualitative reactions for tryptophane in 0.1 per cent solutions of diphtheria toxin, and Pappenheimer finds that a 1 per cent solution gives strong reactions for tryptophane. Diphtheria toxin contains 1.14 per cent of this amino acid as compared with 0.4 per cent in ricin.

With the possible exception of botulinus toxin, the endotoxins of the *Salmonella*, colon, and dysentery groups and certain of the toxic products of the hemolytic streptococcus, bacterial toxins are destroyed by digestion with proteolytic enzymes. This signifies that peptide linkages similar to those found in proteins are essential to the activity of the toxin. It does not necessarily mean that the chemical group or groups in toxins which poison living cells are polypeptide chains. The numerous examples of enzymic destruction of toxins will not be reviewed in detail; some are referred to in other sections of this paper, but the resistance of certain toxins to digestion will be discussed more specifically here.

The resistance of botulinus toxin to digestion has been demonstrated by Bronfenbrenner and Schlessinger (1924), Schubel (1923), Tani (1934), and others. Nelson (1927) found the toxin to be intimately associated with a globulin from the bacterial cell. Digestion with pepsin removed this globulin without destroying the toxin. Botulinus toxin may not be, however, absolutely refractory to digestion. The fact that the minimal lethal dose by mouth is about a hundred times as great as by injection indicates either that much of the toxin is destroyed in the gastrointestinal tract or only a small part is absorbed. In at least one instance partial destruction by pepsin has been observed (Tani, 1934). Karrer (1924) found that ricin is digested very slowly by trypsin. Of a 50 gm. lot of the toxin only two-thirds, as determined by parallel measurements of amino nitrogen and toxicity, was digested by 40 cc. of pancreatic juice in five months.

Resistance to tryptic digestion in the case of the endotoxins of the *Salmonella*, colon, and dysentery groups of organisms is

attributable to the fact that these substances are complex compounds of carbohydrate and lipid. This may also be true of one of the scarlatinal toxins.

### PART III. THE CHANGES PRODUCED BY THE ACTION OF VARIOUS PHYSICAL AND CHEMICAL AGENTS ON TOXINS

The demonstration that a given chemical or physical treatment detoxifies a toxin is of little significance unless the nature of the changes produced in the toxin can be determined or deduced from the experiment. We shall consider here only those studies in which effects on other properties besides toxicity have been examined.

#### *1. Denaturation*

Most toxins are destroyed by the action of heat, strong acid, or alkali. From observations on crude unconcentrated toxins, the nature of the changes induced is not clear. It has been assumed that the lability of some toxins to heat is analogous to the heat-lability of many proteins. The demonstration that purified and concentrated diphtheria toxin is coagulated by heat and denatured by acid and alkali lends support to the view that the destruction of toxins by these agents is actually a denaturation of protein.

Although little is known about the chemical changes which occur when proteins are denatured, it is possible to correlate alterations in biological properties with denaturation. Partial denaturation, without loss of solubility at pH 7.0, apparently affects first those properties of diphtheria toxin designated by Ehrlich as the "haptophore groups." If the denaturation is carried farther, the protein is coagulated and all of the biological properties of the toxin are destroyed. According to the degree of denaturation, diphtheria toxin loses in various degrees its ability to combine and flocculate with antitoxin, its antigenicity, and its toxicity, as shown by Eaton (1936b). An increase in the time required to flocculate with antitoxin is probably the most sensi-

tive indicator of denaturation, and increased Kf<sup>2</sup> is apparent before changes in solubility or other gross evidence of denaturation. The length of time toxin is exposed to denaturing agents affects in a corresponding degree its Kf and antigenicity. Denatured toxin or toxoid of low antigenicity may be separated from unaltered toxin or toxoid of higher antigenicity by chemical treatment of partially denatured preparations. Papers bearing on this subject have been reviewed previously (Eaton, 1936b, 1937b).

The loss of toxicity which accompanies denaturation may be considered either as a decomposition of the toxic groups or as an impairment of the ability to combine with or attack susceptible cells. Possibly heat and high concentrations of hydrogen or hydroxyl ions destroy the toxic groups and at the same time denature the protein and destroy its antigenic properties. It appears, however, that at lower temperatures in neutral or slightly alkaline solutions the rate of toxoid formation or modification of the toxicity is more rapid than the rate of denaturation, while at higher temperatures, and especially in pH ranges above 8.0 and below 5.0, denaturation is more rapid than formation of toxoid. The range of pH for maximum stability varies, of course, for different toxins. Diphtheria toxin is most stable in neutral solution, while botulinus toxin is apparently most stable in acid solution and relatively unstable in an alkaline solution.

The so-called reversible detoxification of diphtheria toxin by acid, as reported by earlier investigators, is probably the result of a combined precipitation and coagulation. Later investigations have shown that the acid-detoxification is only partially reversed by bringing the pH of the solution back to neutrality. It is also possible that the acidified toxin solution precipitates proteins at the site of injection, thus hindering the spread of the toxin.

## *2. Agents which produce destruction, denaturation, or modification*

Many reports dealing with the action of chemicals on bacterial toxins have failed to state what effects were produced on the

<sup>2</sup> Kf is the time required for flocculation of a mixture of toxin and antitoxin in optimal or equivalent proportions. Since this depends on the concentration of toxin and antitoxin in the equivalence mixture, the Kf of different preparations of toxin must be compared by using solutions with the same number of Lf units per cc.



antigenicity or combining power for antitoxin. A review of the earlier work has been published by Bacher (1927). More recently Schmidt (1932) has carried out an extensive investigation of the effects of many aliphatic, aromatic, and alicyclic compounds on the toxicity and Lf value of diphtheria toxin. Only formaldehyde, acetaldehyde, glyoxal, glucose, furfural, hexamethylene tetramine, and certain halogenated hydrocarbons reduced the toxicity without a corresponding diminution in the Lf values. All the other compounds studied either were inert or diminished the Lf in parallel with the toxicity. Judging from the results of Schmidt and other investigators, it seems that the effect of most chemical compounds on bacterial toxins is very similar to that of heat. Many of the substances which destroy toxins also denature proteins.

Von Groer, Altenberg, and Lille (1935) have reported that diphtheria toxin is destroyed by the ortho- and para-dihydroxyphenols but not by the meta-compound. The authors imply that there may be a stereochemical relationship involved, but the o- and p-compounds are much more reactive with a variety of substances than is the m-compound. Schmidt (1932) found that most of the aromatic phenols rapidly destroy diphtheria toxin.

Many of the toxins from anaerobes are quite readily destroyed by oxidation. Diphtheria toxin is not particularly sensitive to mild oxidation or reduction (Hewitt, 1930). Scarlatinal toxin may be in part modified to toxoid by oxidation. Cowles (1936) has shown that cysteine catalyzes an oxidative modification of tetanus toxin. Atmospheric oxygen is used up in the process. The detoxified preparations are antigenic and retain 20 to 50 per cent of their combining power for antitoxin. The work of Halter (1936) indicates that tetanus toxin, when it is diluted in sodium chloride solution, may be destroyed by oxidation. The recent observation of Jungeblut (1937) that about 2 M.L.D. of tetanus toxin may be inactivated by ascorbic acid in amounts between 0.5 and 10 mg. is possibly also connected with an oxidative change. Lippert (1935) finds that methylene blue in the presence of light brings about the destruction of tetanus toxin. He believes that the reaction is an oxidation.

Studies of the photodynamic action of methylene blue on other toxins have been reported by Lin (1936) and Li (1936). Lin states that diphtheria toxin modified by the photodynamic action of methylene blue is apparently more antigenic than formol-toxoid. However, the detoxification was not complete, and, since eight injections were used for immunization, a considerable immunity could have been produced by the residual toxin. No reports of the effect on flocculation were given. Li was able to remove completely the hemolytic, dermo-necrotizing, and lethal properties of staphylococcus toxin by the combined action of methylene blue and light. The toxoid thus produced was equal in antigenicity to formol-toxoid and alum precipitated formol-toxoid.

### *3. The action of formaldehyde*

Formaldehyde apparently acts directly on the toxic groups without affecting other parts of the toxin molecule which are concerned with antigenicity and combining activity. The nature of the chemical reaction or series of reactions that occur may be successfully studied only with highly purified toxin because many other substances in crude toxin also react with formaldehyde.

Diphtheria toxin seems best suited for studies on the process of toxoid formation, because it is easy to measure the amount of antigenic material by the flocculation test and because the toxin may be obtained in a relatively pure state. Bunney (1931) reported that diphtheria toxin purified by acid precipitation could not be detoxified by formaldehyde without destroying the antigenic properties. Others have not confirmed Bunney's results. Eaton (1937c) observed that an excess of formaldehyde in an alkaline solution impairs the flocculating, combining, and immunizing properties of purified toxin during modification to toxoid. The presence of small amounts of nitrogenous impurities in partially purified toxin will markedly affect the action of formaldehyde and prevent destruction.

For detoxification in a solution at pH 6.0 there is required a concentration of formaldehyde one hundred times as great as that sufficient to modify purified toxin to toxoid in a solution at

pH 8.6. Follensby and Hooker (1936), using diphtheria toxin partially purified by acid precipitation, showed that the reaction between formaldehyde and toxin to form toxoid has the characteristics of a unimolecular reaction. The velocity constant of the reaction is stated to be directly proportional to the concentrations of hydroxyl ions and formaldehyde.

Hewitt (1930) has pointed out that the reaction between formaldehyde and toxin to form toxoid is slow and irreversible while that between formaldehyde and the free amino groups of proteins, polypeptides, or amino acids is rapid and reversible. With both crude and purified toxin the amount of formaldehyde required for detoxification in a reasonable time is not greater than the theoretical quantity necessary to combine all of the free amino nitrogen (Eaton, 1937c). In the reaction which occurs in the Sørensen titration a large excess of formaldehyde (about 60 times the theoretical amount) must be added to combine with all of the amino groups. From these facts it is obvious that the reaction which occurs in toxoid formation is not the ordinary reaction between formaldehyde and amino groups.

Compounds of formaldehyde or acetaldehyde and ammonia form toxoid more slowly than do the free aldehydes. Aldehyde bisulphites modify diphtheria toxin to toxoid slowly and incompletely (Eaton, 1937a). Wadsworth, Quigley, and Sickles (1937) have observed that the addition of histidine in a quantity sufficient to combine all of the formaldehyde in a mixture with toxin prevents the formation of toxoid. Apparently the affinity of the toxic group for formaldehyde is greater than that of ammonia or amino groups but less than that of the bisulphite ion or the imidazole group of histidine.

Purified diphtheria toxoid contains about two-thirds of the free amino nitrogen found in purified toxin, and the bound amino nitrogen in toxoid is not liberated by removal of the free formaldehyde (Eaton, 1937c). Wadsworth, Quigley, and Sickles (1937) were able to detect only minute amounts of amino nitrogen, or none at all, in diphtheria toxin partially purified by ultra-filtration. Pappenheimer (1937) found about 1.2 per cent, and Eaton found over 2.0 per cent of free amino nitrogen in their purified

preparations. In the experiments of Wadsworth, Quigley, and Sickles about one-tenth of the formaldehyde from a 0.22 per cent solution disappeared during the complete conversion of toxin to toxoid over a period of 20 days. This quantity of formaldehyde appears to be greater than the equivalent of all the amino groups in both toxin and impurities.

The combination of formaldehyde and amino groups is, of course, not necessarily the reaction concerned in detoxification. Present data are not accurate enough to indicate whether all of the formaldehyde used up during detoxification combines with amino groups, but measurements of this sort are not impossible with highly purified and concentrated toxin. Follensby and Hooker (1936) suggest that the formation of toxoid from toxin may be a reaction catalyzed by formaldehyde and hydroxyl ions. If this is true, the disappearance of formaldehyde to combine with amino nitrogen and other groups is only an incidental reaction. Recently Goldie (1937) has studied the action on crude and partially purified diphtheria toxin of ketene, an acetylating agent that combines directly with free amino groups. In the various samples of crude and of purified toxin 30 to 50 per cent of the amino groups were combined after 10 to 25 minutes' action of the ketene, and the toxicity had been reduced to 1/6 to 1/300 of the M.L.D. in the original sample. At this stage of the acetylation the toxin flocculated with antitoxin, indicating that a partial modification of toxin to toxoid had occurred. With more prolonged action of the ketene, further combination occurred and both flocculating ability and toxicity were destroyed.

Formaldehyde-toxoid is more stable to denaturation than toxin (Eaton, 1937c). It is not at present known whether the acquirement of increased stability is directly connected with loss of the toxic properties. Other proteins also become less subject to denaturation, as judged by decrease in solubility, after the action of formaldehyde. The changes which occur are not understood as yet. The fact that purified toxin and toxoid have the same optical rotation suggests that the optically active atoms adjacent to the peptide linkages may not be affected in the change to toxoid.

With certain proteins, such as casein, formalin produces polymerization, and some investigators have taken the view that toxoid is a polymerization product of toxin. However, in the case of diphtheria toxin, one Lf unit of toxin is modified by the action of formaldehyde to one Lf unit of toxoid, and there is no appreciable change in the nitrogen per flocculating unit. Consequently, polymerization of two or more molecules of diphtheria toxin to form toxoid could only be possible if all the groups which combine with antitoxin remained free. In the case of staphylococcus toxin, the formation of toxoid by the action of formaldehyde is accompanied by a reduction in the combining power for antitoxin to approximately one-half. An indication of a difference in the combining powers of diphtheria toxin and toxoid is found in the work of Madsen, Jensen, and Ipsen (1937) who studied the combination *in vivo* of injected toxin or toxoid with antitoxin in the blood of actively and passively immunized animals. Their results indicate that diphtheria toxin binds twice as much antitoxin *in vivo* as *in vitro*, while toxoid binds the same amount of antitoxin *in vitro* as *in vivo*.

Although denatured toxin of less combining power and toxicity may be separated by chemical means from a mixture with unaltered fully active toxin, the separability of toxin from toxoid has never been demonstrated. The progressive formation of toxoid from toxin may be a process affecting step-wise all the molecules of toxin at once so that there is not at any time an equilibrium between "completely toxic" toxin and "completely atoxic" toxoid. The fact that the complete reaction is irreversible points against the existence of such an equilibrium. There may however, be a reversible equilibrium between molecules modified to different degrees before complete detoxification. This is indicated by some observations of Wadsworth, Quigley, and Sickles (1937) who found that, following the removal of formaldehyde by ultrafiltration from partially modified toxin, the toxicity increased when the preparation was incubated but not when it was kept in the cold room. This implies a partial reversal of the reaction at an intermediate stage.

#### *4. The action of soaps, lipids, and sterols on bacterial toxins*

Vincent (1926) observed that 0.2 to 1.0 per cent solutions of bile and soaps neutralize several hundred M.L.D. of tetanus and other toxins. This author termed the effect a masking of toxicity rather than an inactivation because the reaction was partly reversible. Precipitation of the palmitic acid of the soap with hydrochloric acid liberated enough tetanus toxin to kill guinea pigs, but the killing doses were considerably larger than the M.L.D. of the original toxin.

Larson and Nelson (1924) attributed the detoxifying effects of sodium ricinoleate on diphtheria and tetanus toxins to the property of this substance of forming colloidal aggregates capable of adsorbing other colloids. Larson and Halvorson (1925) observed that the toxin-soap mixture was dissociable. Dilution caused it to become toxic and the firmness of combination increased with time. Bayliss (1936) found that sodium ricinoleate and sodium chaulmoograte are the most effective detoxifying soaps, a 1 per cent solution neutralizing about 35 M.L.D. of diphtheria toxin. Other soaps of chemical composition similar to the ricinoleates and chaulmoogrates were less effective. Unsaturated soaps were generally more active than salts of the saturated fatty acids; bile salts were least effective.

A somewhat different effect of the salts of fatty acids on diphtheria toxin has been observed by Schmidt (1932). The lower members of the series are inactive but, beginning with the fatty acid containing eight carbon atoms in the chain, a destruction of toxicity, flocculating, and immunizing properties is produced by solutions as dilute as hundredth normal acting over a period of several weeks. In these experiments the effect of pH was controlled. Schmidt also observed that a large excess of ricinoleate was necessary to detoxify diphtheria toxin in 24 hours. Contrary to the results of Larson and his collaborators who claimed that their preparations were good antigens, Schmidt states that ricinoleate-toxin has only weak antigenic properties which may be due to traces of free toxin.

Fixation of toxin at the site of injection as a result of previous

adsorption on colloidal particles has been advanced by Ramon and his collaborators (1937b) as the explanation for the neutralizing effects of lanolin on diphtheria and tetanus toxins. By emulsification with 3 or 4 grams of lanolin, 40 M.L.D. of diphtheria toxin or 200 M.L.D. of tetanus toxin were rendered innocuous. Addition of cholesterol diminished the neutralizing effect of lanolin on tetanus toxin. Removal of the lanolin by extraction with acetone, toluol, or chloroform liberated part of the toxin. Mixtures harmless to guinea pigs were found to be toxic for rabbits. Local fixation of the toxin injected with lanolin was demonstrated by studies on the rate of distribution of the toxin in the body of the animal. One or two injections of the toxin-lanolin emulsion are said to produce better immunity than the injection of an equivalent amount of anatoxin. Ramon's results have been confirmed by Eisler and Gottdenker (1937). Using a solution of cholesterol in olive oil, these investigators found that the degree of detoxification of diphtheria toxin depends on the relative volumes of toxin and oil and the length of time these are shaken together. The results apparently depend on surface effects in the droplets of oil. Aqueous emulsions of cholesterol do not affect diphtheria toxin. According to Ramon cholesterol is less effective than lanolin as a detoxifying agent. Schwartz (1936) has reported that the hemolytic, necrotizing, and lethal properties of staphylococcus toxin are markedly diminished by shaking with olive oil.

The colloidal phenomena just described differ in several ways from the strictly chemical effect of formaldehyde on toxin. Relatively enormous amounts of the soap or lipid (in the proportion of about 100,000 parts to 1 of toxin) are required to detoxify. The effects on the antigenic and combining properties of the toxin are not at present clear. Soaps apparently affect the properties of the toxin in a way which is similar in part to denaturation and in part to combination with antitoxin. Possibly partial denaturation of toxin is produced by surface effects in emulsions just as shaking with air denatures purified and concentrated diphtheria toxin (Pappenheimer, 1937), and proteins generally. On the other hand, some of the effects are at least

partially reversible. The high antigenicity of lanolin-toxin mixtures seem to preclude denaturation. Probably some of the agents act by delaying absorption of the toxin at the site of injection. Others, such as soaps, may act by binding those parts of the molecule which attach the toxin to susceptible cells, antibody-producing cells, or antitoxin. True modification of toxin to toxoid probably does not occur in any case.

5. *The effects of various chemicals on toxins and other substances that act on red blood cells*

Substances that hemolyze or agglutinate red cells are of interest in connection with the chemistry of toxins, because factors affecting the combination of active substance and susceptible cells may be studied. Staphylococcus toxin and plant toxins such as ricin also produce toxic effects *in vivo*. Others acting only *in vitro* are pneumococcus hemolysin, tetanolysin, and the crystalline protein concanavalin A (Sumner and Howell, 1936), which agglutinates red cells and precipitates a variety of carbohydrates and lipids.

Hypertonic salt solutions inhibit hemolysis by immune serum and complement and also by bacterial hemotoxins. Rigdon (1937) finds that the combination of staphylococcus hemotoxin with rabbit red cells is prevented by 6 per cent sodium chloride solution. Avery, Rigdon, and Johlin (1937) report that magnesium sulphate and several salts of sodium and potassium inhibit hemolysis by staphylococcus toxin. The production of skin necrosis by this toxin is also inhibited by hypertonic solutions of sodium chloride, magnesium sulphate, and lithium chloride. The latter salt, however, does not prevent hemolysis. Smith (1937) has found that glycerol, ethylene glycol, sucrose, and glucose, diminish the necrotizing and lethal properties of staphylococcus toxin but have no effect on the titration of toxin by hemolysis of red cells. Weinstein (1937) reports that the hemolysis produced by colonies of streptococci and staphylococci growing on blood agar is inhibited by adding lecithin to the medium. Cholesterol prevents this inhibition of hemolysis. This recalls antagonistic action of cholesterol on the neutralization of tetanus



toxin by lecithin as observed by Ramon. Salts, lecithin, and polyhydroxy-alcohols may act directly on the hemotoxin, or they may alter the surface of the cells so as to prevent attachment of the toxin.

The identification of at least one chemical group associated with the activity of pneumococcus hemotoxin has been accomplished by Cohen and Shwachman (1936), and Shwachman, Hellerman, and Cohen (1934). Pneumococcus hemolysin is inactivated by oxidation and reactivated by reagents that can restore free thiol groups; therefore the lytic activity is associated with the presence of sulfhydryl groups in the preparation. Reversible oxidation is produced by a variety of agents, and zones of reduction potential in which the hemolysin is active or inactive have been defined. The iodoacetate ion which inactivates certain enzymes also inactivates the hemolysin, but does so reversibly.

Cohen, Shwachman, and Perkins (1937) have examined the effects of various sterols on pneumococcus hemolysin. Irreversible inactivation is produced by cholesterol and coprostenol which contain double bonds and are precipitable by digitonin. The saturated sterol, coprostanol, is less active and various sterols not precipitated by digitonin are practically without effect. Binding of the hydroxyl group by esterification as in cholesteryl acetate also removes the inhibitory action. These observations are similar to previous ones on tetanolysin and saponin. Diphtheria toxin differs from these substances in being neutralized both by sterols and their esters. This indicates that a different mechanism is involved (Eisler and Gottdenker, 1937).

Active pneumococcus hemolysin combines rapidly with red cells and with cholesterol. Inactive hemolysin does not combine with red cells nor, apparently, with cholesterol, because after treatment with the latter the hemolysin may be reactivated as usual. It remains to be seen whether or not this indicates that the active hemolysin combines with cholesterol in the red cells. Should it do so, then the attachment could not occur through the sulfhydryl groups because these apparently remain free

(positive nitroprusside reaction) in the hemolysin after inactivation with cholesterol. At any rate, the state of oxidation of the sulfhydryl groups apparently conditions the affinity of other, unknown groups in the lysin for sterols and for red cells. The actual lysis of the red cells may be due to enzymic properties of the hemolysin. The loss of hemolytic power is apparently not accompanied by a loss of the ability to combine with antibody. Hull (1936) found no change in the combining capacity for antibody of hemolytic extracts of the pneumococcus after storage in the ice-box for a length of time sufficient to cause a marked decrease in the hemolytic property.

6. *The effect of vitamin C (ascorbic acid) on diphtheria toxin in vitro and in vivo*

The effect of vitamin C on poisoning with diphtheria toxin has been studied extensively since Greenwald and Harde (1935), and Jungeblut and Zwemer (1935) observed that feeding vitamin C to guinea pigs increased their resistance to 1 or 2 lethal doses of toxin. The effect of vitamin C on toxin *in vitro* is of doubtful significance. The investigators just cited reported that up to 10 M.L.D. of toxin were inactivated by 10 mg. of ascorbic acid per M.L.D. (The M.L.D. of pure diphtheria toxin is 0.0001 mg. or less.) Grooten and Bezssonoff (1936) state that 100 mg. partly detoxify 4 M.L.D. Hanzlik and Terada (1936) observed that neutralization of toxin was absent or irregular in alkaline solutions of vitamin C; and they found no protection of pigeons by vitamin C against diphtheria toxin. Torrance (1937b) reports that heated and unheated crude toxin catalyzes the oxidation of vitamin C in lemon juice, an effect that may be due to porphyrins in the crude filtrate. Lemon juice has no effect on the toxicity or Lf value of the toxin. Sigal and King (1937b) find that properly neutralized and buffered solutions of vitamin C do not inactivate diphtheria toxin when oxidation is inhibited by diethylthiocarbamate. The effects of vitamin C on diphtheria toxin *in vitro* may be attributed to oxidation and acidity.

The injection of sublethal doses of diphtheria toxin in guinea pigs causes a depletion of vitamin C from the suprarenals, pan-

creas, and kidneys (Lyman and King, 1936; Torrance, 1937a; Haas, 1937b). Lyman and King observed an increase or a decrease of vitamin C in the liver, depending upon the dose of toxin and the amount of vitamin given daily. Torrance reports that injection of small amounts of toxin causes a mobilization of vitamin C in the suprarenal gland, but Haas was unable to confirm this. Sigal and King (1937a) have studied the mode of action of vitamin C in diphtheria intoxication. Injection of sublethal doses of toxin into animals on a diet deficient in vitamin C produced a degeneration of the islets of Langerhans, a hyperglycemia, and a low glucose tolerance. The effect was less marked in animals on an adequate diet, but the amount of vitamin necessary for a maximum effect on the intoxication was much larger than that necessary to protect against scurvy and maintain the normal growth rate.

#### CONCLUSION

The last five years have witnessed definite advances in our knowledge of the mode of production and the nature of bacterial toxins. The development of media containing relatively simple nitrogenous substances of known composition, such as those used for the cultivation of *Corynebacterium diphtheriae* and *Staphylococcus aureus*, will undoubtedly make possible more exact chemical studies of toxins. The complex conditions under which toxins are formed can be worked out most easily when the constituents of the media are known. Among the important factors affecting the formation of toxins are conditions related to oxidation-reduction systems. Many other factors have not yet been clearly defined in their relationship to the mechanism of toxin formation.

The ease with which various toxins are liberated from the bacterial cells differs greatly. Some toxins such as that of *C. diphtheriae* appear to be secreted in a readily soluble form by the bacteria. Others are liberated only by death and autolysis of the cells. The view that toxins are formed by enzymic degradation of proteins or peptones in the culture media is becoming less tenable as more toxins are produced in media containing

substances not much more complex than the amino acids. It is possible, however, that some of the bacterial poisons, such as the enterotoxin of the staphylococcus and the hemolysin of the streptococcus, may be metabolites or products of the action of bacterial enzymes.

At the present time, the only bacterial toxin which has been isolated in a state approaching purity is diphtheria toxin. The concentration and partial purification of others by various chemical procedures has assisted in the study of bacterial products with poorly defined biological properties. Chemical studies of the toxic products of the hemolytic streptococcus have led to the discovery that this organism forms a host of substances, each having some of the characteristics of a toxin. In the separation of the endotoxin from the exotoxin of the Shiga dysentery bacillus it was shown that the gastro-enteric endotoxin is a carbohydrate-lipid complex, while the neurotropic exotoxin is a protein. This is an advance which may eventually enable us to differentiate endotoxins from exotoxins on a chemical basis.

Attempts to demonstrate the existence of a toxic group in toxins by chemical analysis have so far been unsuccessful because of the difficulty of obtaining toxins in a pure state and in sufficient quantity. Both ricin (a plant toxin) and diphtheria toxin appear to be proteins which cannot be further degraded without destroying their biological activity. There is at present no evidence that these are conjugated proteins containing a prosthetic group which is responsible for their physiologic activity. However, it is possible that other toxins, such as those of *Streptococcus hemolyticus*, *Clostridium botulinum*, and the endotoxins of the *Salmonella*, colon, and dysentery groups, may prove to be non-protein substances with some unique chemical configuration which gives them their toxic properties.

Studies on the action of various physical and chemical agents on toxins have begun to yield suggestive data. Toxins are apparently affected in three different ways: (1) by agents which produce denaturation or coagulation, (2) by agents (soaps and lipids), which reversibly mask the toxic and biologic properties, and (3) by agents (certain aldehydes, halogen compounds, and oxidizing

agents) which produce modification to toxoid. Further investigations with purified toxins and chemical reagents classified into these three groups will doubtless yield important information on the nature of toxins.

Many toxins produce pharmacologic effects resembling those of the alkaloids. Toxins differ, however, from ordinary poisons in their tremendous activity and in the properties connected with antigenicity and the ability to combine with antitoxins and with susceptible cells. The association of antigenicity and great toxicity may be more than incidental. It is not unlikely that the same property or chemical grouping which causes an immunologic response when the toxin is injected may also bring about a selective combination with susceptible cells, just as an enzyme combines selectively with certain substrates. The pharmacologic effect would then follow by the action of other parts of the toxin molecule. From this it would appear that the nature of toxins as proteins or protein-like substances may hold the secret of their most characteristic biological properties.

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<sup>4</sup>In preparing this review, the author has not attempted to cover every paper having a bearing on the subject. Enough references are given to illustrate each point but there are many other papers on similar work which have not been cited. Generally the most recent papers have been reviewed without regard to historical development or priority. The papers cited usually contain adequate references to earlier work.

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# SEROLOGICAL RELATIONS AMONG SPORE-FORMING ANAEROBIC BACTERIA

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Immunochemistry in recent years has offered new proofs and better criteria for the specificity of antigen-antibody reactions (134, 174, 175, 207). These advances have strengthened the position of serologists by providing a firmer basis for their examination of biological materials, such as bacterial constituents. Remarkable examples of group- and species-specificity have been found, and in certain instances the information has been useful in bacterial classification.

It is the purpose of this review to present and evaluate the serological data relating to the spore-forming anaerobes<sup>1</sup> and to discuss the relationships disclosed and their bearing upon the taxonomy of these organisms. One must admit, however, that the serological relationships are not always in harmony with the present taxonomic schemes. Examples will be given in which the serological evidence may require consolidation of species that are at present listed separately. In other instances, the evidence either confirms the present species or reveals subdivision of them. For the present, then, it can only be claimed that the serological approach has discovered a certain measure of order, and that it does aid in the differentiation of the closely related spore-forming anaerobes.

The subject-matter of this review will be discussed under the

<sup>1</sup> Comparatively little is known concerning the serology of the non-spore-forming species, although several papers on this topic have appeared lately: 9-13, 59-61, 79, 80, 145, 303, and 364.

headings: *a* Toxigenic anaerobes and their toxin specificities (including the problem of atoxic strains); *b* Agglutination reactions; and *c* Precipitin and complement fixation reactions.

#### THE TOXIGENIC ANAEROBES AND THEIR TOXIN SPECIFICITIES

The toxins produced by certain of the spore-forming anaerobes have long been recognized as specific substances, though of unknown composition. Physiological evidence of this specificity is found in the consistent action in the animal body of such toxins as tetanospasmin and the neurotoxin of *Clostridium botulinum*.<sup>2</sup> The specificity of the toxin-antitoxin reaction was employed in the recognition of an anaerobe by Roux as early as 1888 (271). Through the World War period, when discovery of new anaerobes was abnormally stimulated, it became almost a routine to test for toxin neutralization with antitoxins of the most probable group relatives before a newly isolated strain was identified or described as new (52, 55, 168, 200, 277, 359, 360). In polymicrobial infections, like gas gangrene, proof of the etiologic agent was often obtained by "animal protection experiments" (70, 144, 176, 177, 263, 330). And finally, the controversy as to whether "Rauschbrand" of animals was comparable to gas edema in man, and caused by the same organism, was settled largely by study of the toxin specificities of *Clostridium chauvoei* and *Clostridium septicum* (55, 107, 169, 178).

Partly because the toxins are usually produced in complex nitrogenous media, the problems of the purification and nature of these substances are difficult to solve. Nevertheless, considerable efforts are being made to develop methods for purifying,

<sup>2</sup> Throughout this paper problems of nomenclature have been ignored so far as possible. In general, the name first proposed has been employed in direct reference to the discovery of an organism; in section headings and in later discussion that name, as adapted to placement in the genus *Clostridium*, has been used for all commonly accepted members of the genus. For the less familiar *Clostridium* species and particularly those whose familiar names have thus been displaced, synonyms have been given in parentheses following the first citation of the species. Whenever inclusion of an organism in *Clostridium* would involve the creation of a combination of names new to the literature, the transfer has not been made; in such cases, the original name in *Bacillus* has been retained for clarity of reference. This policy has seemed to the authors preferable to debating and deciding specific problems of nomenclature on this occasion.

preserving, and characterizing the principal toxins and antitoxins (19-24, 33, 78, 101-104, 129, 148, 152-154, 158, 204, 205, 290, 293, 298-300, 312, 313, 323, 336, 337, 339, 371). Recently two agencies, the Inter-governmental Conference on Biological Standardization and the Permanent Standards Commission of the League of Nations Health Organization, have undertaken to define international units for toxin and antitoxin preparations for tetanus and the gas gangrene organisms (1, 93, 94, 152, 153, 193, 204, 205, 337). The work of the League Committee has been directed primarily toward the definition of units and the investigation of the accuracy of titrations of toxin-antitoxin preparations. The State Serum Institute at Copenhagen is the depository for the standards. As the United States is not a member of the League, it has no official part in determining the international policy; but it has coöperated in conferences and in making tests. Definitions of the United States equivalents of the International units are made by the National Institute of Health. In some cases, the same units have been adopted, for example, that for oedematiens antitoxin (23); in other cases, a pre-existing American unit has been defined in terms of the International unit, for example, that for tetanus antitoxin (193).

Enough is known about the differentiation of the toxins of the principal anaerobic species to permit a discussion of these specific substances as a basis for the typing of the toxigenic organisms.

*The toxins of Clostridium tetani, Clostridium septicum, and Clostridium histolyticum*

*Clostridium tetani* presents a comparatively simple case of toxigenicity. Its toxin was discovered by Kitasato in 1889 (166), and the specific antitoxin by von Behring and Kitasato in the following year (15). The pharmacological action of the toxin indicates the presence of at least two factors: tetanospasmin and tetanolysin (87, 118, 181, 203, 258, 326, 335). For purposes of production of antitoxin, however, no distinction of these factors is made, the cell-free filtrate of a culture being used either directly, or modified by iodine (329), formol (278), or in combination with alum, tapioca, lanolin, etc. (255-257). Furthermore, experience has shown that a protective serum can be made



from any strain of true *C. tetani*. For this reason the toxin is called monotypic, a point of particular interest in view of the subdivision of the species into serological types by other reactions. The question of the mode of absorption and action of tetanus toxin has recently been reopened; but there is not as yet agreement upon the route of transfer of the toxin to the central nervous system (2-6, 74, 90).

*Clostridium septicum* (*Vibrion septique*; *Bacillus oedematis maligni*) also produces a toxin which has no type-specificity, although sub-types on the basis of agglutination do exist within the species. The general serological properties of the toxin are known (19, 20, 71, 158, 264, 266, 338, 348). The pathology of the extremely rapid death of animals injected intravenously with the toxin has been explained recently (245). A specific action of the toxin on the heart muscle, causing Zenker's degeneration, is the immediate cause of death, although lesions also occur in the kidney, spleen, and other organs.

*Clostridium histolyticum* also produces a toxin which is monotypic for the species but is apparently a complex of myolytic and hemolytic factors. The organism is unique in its myolytic action, a property which has been made use of in the Connell method (54) for the lysis of cancerous tissue. The efficacy of this treatment has been denied (117, 250, 272); but its proposal has at least led to a more thorough study of the proteolytic enzyme system, upon which the myolytic action probably depends (309, 340). It is peculiar that the red blood corpuscles remain intact in the residue of lysed tissue (123, 362). In culture, hemolysin is produced under proper conditions, but not in proportion to the general potency of the toxin as determined by the minimal lethal dose (228, 309). The preparation of the toxin and antitoxin and their standardization have recently been investigated (24, 153, 337).

*The toxin of Clostridium oedematoides (Clostridium oedematis sporogenes; Bacillus sordellii)*

A new species of the gas-gangrene group was discovered by Sordelli in 1922 (301). Because of its resemblance to *Clos-*

*tridium oedematiens* in pathogenicity in a human case and to *Clostridium sporogenes* in culture, it was called *Clostridium oedematis sporogenes*. The name, being a trinomial, was objectionable and was changed to *Bacillus sordellii* by Hall and Scott (127). Meanwhile, Meleney, Humphreys and Carp (219) discovered a *Clostridium oedematoides*, resembling *C. oedematiens* and *C. septicum* but distinguishable from both by toxin neutralization tests. The following year the toxins of *C. oedematoides* and *B. sordellii* were found to be identical; *C. oedematoides* was declared invalid and *B. sordellii* considered the preferred name for the species (125, 150). Agglutination tests have also indicated that the two organisms are identical (126). However, since *Clostridium* is now the widely accepted genus for the spore-forming anaerobes, *C. oedematoides* would be valid, and it has been retained by Hauduroy *et al.* (131) in the new French "*Dictionnaire des bactéries pathogènes pour l'homme, les animaux et les plantes.*" Nevertheless, the combination: *B. sordellii* or *Clostridium sordellii*, according to the choice of the user, continues to be in favor in this country.

Further study of the toxin-antitoxin reaction of this species has disclosed an unforeseen relationship to another anaerobic species. In the course of their study of *Bacillus bifermentans*, Clark and Hall (47) discovered positive but "weak" cross-agglutination between *B. bifermentans* and *B. sordellii*. Moreover, the serum of rabbits immunized with *B. bifermentans* was found to be protective to guinea pigs injected with toxin of *B. sordellii*. However, since the pathogenicity of *B. sordellii* contrasts sharply with the non-pathogenicity of *B. bifermentans*, Clark and Hall did not recommend combining these species. Spray (304) has also respected the difference in pathogenicity and has made it the only point of separation in his key to the spore-forming anaerobes. The relationship has been further investigated by Stewart (310), who has found 1 cc. of an experimental antiserum for *C. bifermentans* to protect mice against 2 to 5 M.L.D. of the toxin of *C. sordellii*. Positive agglutination and precipitation reactions have also been obtained, thus linking the two species. Because they are also indistinguishable in

morphology, colony formation, and biochemical reactions, consolidation has been recommended under the name: *C. bifermentans*, which has priority over *C. sordellii*. The antitoxin would then be called by Stewart bifermentans antitoxin, but whether this terminology will come into general use remains to be seen. A somewhat analogous case involving *Bacillus oedematis maligni* II of Novy (now called *Clostridium novyi*) and *C. oedematiens* will be discussed later.

Ictero-hemoglobinuria of cattle in Nevada is caused by an organism which was at first called *Bacillus hemolyticus bovis* and later, *B. hemolyticus* (331, 333). For a time, there was some question whether it also could be identified with *C. oedematoides*, but apparently the latter is merely associated with it in certain cases of the disease (120).

#### *The toxins of Clostridium parbotulinum and Clostridium botulinum*<sup>3</sup>

After recognition by Leuchs (182) of the production of multiple toxins by *Clostridium botulinum*, Burke (45) reported two distinct toxin groups, arbitrarily called Types A and B, in the 12 American strains studied by her. The antitoxin of one type neutralizes the toxin of the homologous but not of the heterologous type. An important contribution by Bengtson (16, 18) revealed a third toxin group, Type C, represented by cultures<sup>4</sup> from larvae of *Lucilia caesar* taken from a chicken, which had

<sup>3</sup> The nomenclature of the botulinus-parabotulinus group is particularly confusing, because of a lack of understanding of their separation on the basis of proteolysis. The terminology was introduced by Bengtson in 1924 (18). As recently as 1937, however, the distinction between *C. botulinum* and *C. parbotulinum* was not appreciated, which unfortunately adds to the confusion (369). It is beyond the scope of this review to argue the nomenclature; the original basis of separation and discussions regarding it may be found in the following articles: 18; 112; 131; 186; 221-224; 268; 288; 315-319; 321, pp. 687-690, 1276-1279; 350, p. 107; 358, pp. 332-340; 369. We have accepted the terminology of Bengtson and have therefore used *C. botulinum* to refer to non-proteolytic cultures found within the toxin types B, C, D, and E. Proteolytic cultures, termed *C. parbotulinum*, have been found thus far only within the toxin types A and B.

<sup>4</sup> The Type C organism has been called *Clostridium luciliae* by Bergey (29) and by Spray (304), but the name has never come into common use and seems not likely to, because it is at variance with the Bengtson nomenclature.

died of limberneck. Probably identical is the organism later isolated by Graham and Boughton (106), and in the same toxin group, at least, is the organism of Seddon (287, 288) from botulism of cattle in Tasmania. Pfenninger (248) showed that the Seddon organism produces a toxin, which can be neutralized by the Type C antitoxin prepared by Bengtson, but that the antitoxin for the Seddon culture neutralizes only its homologous toxin. These observations have been confirmed, and the two factors have been designated  $C_\alpha$  and  $C_\beta$ , within Type C toxin (110, 112, 114). A Type D toxin was added after study of the organism associated with botulism or lamsiekte of cattle in South Africa (224, 315, 316, 319). Finally, a Type E was proposed by Gunnison, Cummings and Meyer (111) to designate the toxin of an organism obtained from Russia and originally isolated from spoiled fish. (Topley and Wilson (321, p. 689) have erroneously cited Type E as the cause of equine botulism in South Africa; in reality (268), the disease is due to *C. botulinum*, Type C.) Apparently the organism of Hazen, which was unfortunately incompletely reported (132), is also a Type E culture.<sup>5</sup> Thus, there are now five types of the botulinus toxin.

The work of Mason and Robinson (216) is of interest because of its extensive experiments on the antigenic components of these toxins. The A and B toxins appear to be monospecific, whereas the C and D toxins are not so simply constituted. It has been claimed that C toxin is composed of two major fractions,  $C_1$  and  $C_2$ , which may vary quantitatively with the conditions of incubation; and that D toxin is present, but in small amount only, in the so-called Type C toxin. Although the Type D organism produces chiefly D toxin, there is also apparently some admixture of C toxin. Confirmation of this distribution of the fractions is needed; and extended study should be made of all available cultures of these types, with particular attention to the conditions for production of the toxins. The new Type E strains and others, which may be isolated from widely separated geographical regions, should be included in the study.

<sup>5</sup> Personal communication from Dr. K. F. Meyer. A paper reporting this finding is in preparation.

*The toxins of the Clostridium welchii group*

From the time of isolation of the organism now called *Clostridium welchii* (*Bacillus aerogenes capsulatus*, *B. phlegmonis emphysematosae*, *B. perfringens*) there have been many reports of its remarkable pathogenic action. The acid (principally the butyric acid) was at first regarded as a tissue irritant and debilitant (49). As late as 1916, it was argued that no true toxin was concerned but that the acid injury was a sufficient cause of the lesions of gas gangrene (307). The mechanical effect of the gas in blocking circulation and contributing to the spread of infection was also considered important (202). The severe toxemia and the rapidly fatal outcome, however, made it seem likely that a potent toxin was involved. In 1917, Bull and Pritchett (42-44) obtained the soluble toxin *in vitro* and prepared the antitoxin. The main properties of the toxin were soon determined; and again a multiplicity of toxin elements was indicated. These have been named after their apparent physiological action: a hemotoxin or hemolysin (42, 46, 88, 142, 157, 242, 280, 357, 374); a myotoxin (143); a neurotoxin (346); another toxin said to act specifically on the blood vessels (347); "necrotic and lethal" toxin (99); the "acute lethal" toxin of Shiraishi (289); and finally the so-called "pseudotoxin" (7, 170), which is heat-stable, non-antigenic, and non-neutralizable by antitoxin. This "pseudotoxin" may be the histamine or histamine-like substances found among the growth-products of *C. welchii* (162). With such an array of toxin elements (though some of them appear to have been proposed on very meagre evidence), it is no wonder that the testing of toxin and antitoxin of *C. welchii* presents considerable difficulty. The proportion of hemolysin, for example, varies widely (357), and this variation interferes with the measurement of the protective power of an antiserum by the titration of its antihemolysin content (99, 143, 215, 252, 253, 354).

Further evidence of the complexity of *C. welchii* toxins appears, when one considers the several organisms resembling, but apparently not identical with, the *C. welchii* of classical gas gangrene. In recent years many reports have claimed, or questioned, the association of *C. welchii* with intestinal disorders of man, such as

flatulent diarrhea (161, 234, 311), or the toxemia of intestinal obstruction (146, 201, 233, 320, 366). Also, in diseases of the enterotoxemic type among domestic animals, there are found organisms resembling *C. welchii*. Lamb dysentery in Great Britain, one of the first of the enterotoxemias to be investigated, is ascribed to the lamb dysentery bacillus of Dalling (62, 92), sometimes called *Bacillus agni*. The bacillus is not identified as *C. welchii* because of minor physiological differences, and because the antitoxin for *C. welchii* is not capable of neutralizing the toxin of the Dalling bacillus, although its antitoxin is capable of neutralizing the toxin of *C. welchii*. However, the hemolysins of the two organisms can be neutralized by either antitoxin. Soon another organism from a sheep disease, called *struck* in England, was discovered by McEwen (195-198). It resembles both *C. welchii* and the Dalling bacillus, but differs in a few physiological reactions, and significantly in its toxin-antitoxin specificity. The toxin of McEwen's organism, *Bacillus paludis*, is not neutralized by the antitoxin of *C. welchii* (human), nor the toxin of *C. welchii* by the antitoxin of *B. paludis*. McEwen inferred, therefore that his organism differed also from Dalling's bacillus, especially since he was not at first able to show that *B. paludis* produces a hemotoxin. Complicating still further this group of *welchii*-like organisms of animal diseases is the organism found in enterotoxemia of sheep in West Australia (25, 27) and the "pulpy kidney" disease of sheep reported from New Zealand (97, 98), Tasmania (26, 244), Australia (26), Palestine (96), and North Wales (229, 230). Apparently the same organism occurs in other herbivorous animals, and it has been considered as the possible etiologic agent in the grass disease of horses (105). Bennetts (25) discovered and described the organism of sheep enterotoxemia in 1932. Again, because of the differential specificity of its toxin, as evidenced in cross-protection experiments with the classical *C. welchii*, *B. paludis* of McEwen, and the Dalling bacillus, Bennetts considered his organism a new species, which he named *Bacillus ovitoxicus*.

Prophylaxis of the various sheep diseases has been tried with both antitoxin and toxoid vaccinations (27, 68, 69, 212). In

experiments of this nature, it is important that the cross-protective powers of the antisera produced for the several species be known, because investigation of the distribution of the diseases has shown that the organisms may occur widely and at random, —*B. paludis* has been reported in France, Greece and Turkey; *B. ovitoxicus* in Scotland, North Wales, Palestine, United States, etc. (25, 27, 64, 96, 231, 355, 356). Studies of the toxin-antitoxin relations were made at the Cambridge Institute of Animal Pathology and the Wellcome Physiological Research Laboratories at nearly the same time. The Wilsdon report from Cambridge was published first, showing clearly four organisms, Types A, B, C, and D, on the basis of toxin-antitoxin reactions (367). Type D was then new in England and, although subsequently shown to be *B. ovitoxicus* Bennetts (368), it is still often called the Wilsdon Type D organism. The toxins and antitoxins of these organisms display considerable overlapping of factors, the details of which as determined by cross-protection experiments are as follows:

ORGANISM	TOXIN TYPE	TOXIN FACTORS	ANTITOXIN NEUTRALIZES
<i>Cl. welchii</i> .....	A	W	A
Dalling's.....	B	WXZ	A, B, C, and D
<i>B. paludis</i> .....	C	WZ	A, B, and C
<i>B. ovitoxicus</i> .....	D	WX	A and D

Wilsdon closes his report with the statement that "although the strains can also be grouped according to one or another of their biochemical characters, it is held that these are of minor importance compared with differences in antigenic structure revealed by toxin-antitoxin reactions."

In 1933, Glenny, Barr, Llewellyn-Jones, Dalling and Ross (100) of the Wellcome Laboratories announced the toxin fractions  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ; to these was added the factor  $\epsilon$ , said to be present in Dalling's early cultures of the lamb dysentery bacillus but weak or lacking in many Type B strains of the present day (211). Prigge (252-254) has recently added a  $\zeta$  factor, so far

known only in the true *C. welchii* or Type A. The properties of these factors in physiological terms are (65, 211, 252):

- $\alpha$ —hemolytic, lethal and necrotic
- $\beta$ —lethal and necrotic
- $\gamma$ —lethal
- $\delta$ —hemolytic
- $\epsilon$ —lethal and necrotic
- $\zeta$ —lethal

The distribution of these factors in the respective toxin types is as follows (65):

TOXIN TYPE	FACTORS					
	$\alpha$	$\beta$	$\gamma$	$\delta$	$\epsilon$	$\zeta$
A	+	—	—	—	—	+
B	+	+	+	±	+	
C	+	+	+	+	—	
D	+	—	—	—	+	

With six factors involved in the toxin complex, instead of the three (WXZ) originally recognized by Wilsdon, problems of cross-protection for the four types of *C. welchii* organisms will require reinvestigation, the more so since five of the six factors are reported to be lethal. Nicholson (235) studied the action of the whole toxins of the Type A, B, C, and D organisms on the circulatory and respiratory systems; but his findings are difficult to interpret for obvious reasons.

The most remarkable organism of the four is Type B, or Dalling's lamb dysentery bacillus, which has at least five of the toxin factors. Its antiserum should therefore protect against the toxins of Types B, C, and D. The discrepancy to be explained is that it also protects against Type A toxin, according to Wilsdon and Montgomerie and Rowlands (232, 367), although the lethal  $\zeta$  toxin of Prigge is not known to be formed by Dalling's bacillus. Similarly Type C antiserum, which should lack  $\epsilon$  antitoxin, protects against Type B toxin, which has the  $\epsilon$  factor. That there is still some fine point of relationship to be worked out between



these two types is probable, because Dalling's laboratory has record of a Type B culture, which has become Type C by loss of the  $\epsilon$  factor (64). The classical *C. welchii* would seem to be the most restricted of all in respect to toxin factors, yet there is still something to be explained in the cross-protective power of its antiserum. Commercial antisera for *C. welchii* are of Type A mono-specificity, as judged by their neutralizing power. Nevertheless, they will protect guinea pigs from infection by Type D organisms, which differ from the Type A organisms in possessing the  $\epsilon$  toxin factor (31, 32).

The suggestion has been made (36) that gangrene antiserum for man might be improved by being made polyvalent, for apparently there is ample opportunity for infection of man by any member of the *C. welchii* group. Thus far, however, Types B, C, and D are known as pathogens of domestic animals only; but they can upon parenteral injection give rise to typical gas gangrene in experimental animals (197a). Very little is known of the distribution of the types of *C. welchii* in the intestinal tracts of man and other animals. Borthwick and Gray (35) have evidence of only Type A in human feces, whether of normal individuals or of a patient with pernicious anemia. In rabbits only Type A has been found and in guinea pigs only Type D (34). In the dog both Types A and D were found, but investigation showed that the newly-isolated Type D strains tended to revert to Type A, which involves only loss of their X factor (Wilsdon's terminology). A further study of the D $\rightarrow$ A conversion would be of decided interest, in view of Borthwick's finding (31) that commercial mono-A type sera will protect experimental animals from either Type A or D infections, although neutralizing only Type A toxin. Prigge's last contribution (254) on the importance of the  $\zeta$  toxin of Type A organisms in relation to the protective power of the sera, should be considered also in the problem of the relation between Types A and D organisms. For the maximum protective power of *C. welchii* antisera, Weinberg (341, 352, 353) has advocated those sera which are both antitoxic and antibacterial. Such sera he would call "holosera" or "anti-

exo-endo-toxic sera." Their advantage has not yet been adequately shown.

Other papers have appeared characterizing the interrelations of these organisms and have confirmed the main facts (31, 72, 122, 211, 217). In one case, cultures derived from single cells were used to dispose of the possibility that the overlapping of toxins and certain irregular proteolytic action of the types might be due to mixture of the closely related organisms (210).

Such is the present state of knowledge of the toxins of the *C. welchii* group. Some points of uncertainty remain: whether the lamb dysentery strain of 1923 possessed the toxin factor for *B. ovitoxicus*, as its serum seemed to indicate (63); why there are discrepancies in hemolysin production by cultures tested at different times (195, 252, 354); how stable the toxin types are, in view of the D→A conversion, and so on. The etiologic agents of sheep diseases in different parts of the world are still being investigated. Recently, for example, the "bloedpens" of South Africa has been identified with the lamb dysentery of Great Britain, and its causal organism also found to be *C. welchii* Type B, complete with the  $\epsilon$  toxin factor (211, 213). Lamb dysentery in Montana is apparently not the same (208). Enterotoxemic jaundice or sheep yellows in Australia is due to *C. welchii* Type A (269). The peculiarities of the enterotoxemias are not yet fully understood. There is an intestinal factor, possibly trypsin, obtained in filtrates of the intestinal contents, which when added to Type D toxin renders the mixture very much more toxic to the mouse (37, 38). A "diffusion factor" in cultures of various anaerobes, including *C. welchii*, has also been reported (184). Until more is known of these factors which apparently affect the permeability of the intestinal walls, it is difficult to evaluate the reports. They are perhaps important in determining the pathogenicity of the *C. welchii* regularly found in the intestinal tract.

Short reviews of the interrelations of the toxins and of the etiology of the various diseases due to the *C. welchii* group are available (64, 72, 373). Discussions at scientific meetings have brought out interesting points, some of which have not been made in other published papers (32, 36, 65-67, 286, 342, 343).

*The toxin of Clostridium oedematiens and its near relatives*

*Clostridium oedematiens* was found by Weinberg and Séguin in certain cases of gas gangrene, occasionally alone but more often associated with *C. welchii*. Its toxicity was proven and it was successfully differentiated from *C. septicum* and *C. welchii* (359, 363). The therapeutic value of the *C. oedematiens* antitoxin has justified its inclusion in the polyvalent sera for gas gangrene (94, 204). Fortunately, the toxin is monotypic, for Weinberg, Nativelle, and Prévot (358) have stated that the antisera will neutralize toxin of all strains, whether of human or animal origin. Toxigenicity is relatively weak, or appears to be so because of the instability of the toxin. The properties of the toxin and the conditions for its preservation have recently been investigated (23, 339).

There remains, however, the question of separation of the toxin of *C. oedematiens* from those of certain of its close relatives. Sacquépée (275, 277) discovered an organism at first called "bacille de l'oedème malin," later *Bacillus bellonensis*. There was for a time doubt of its purity and of its relation to *C. oedematiens* (274, 276, 361), but it has recently been accepted by Weinberg (358) as a separate species with a specific toxin. Sacquépée (277) distinguished it from *C. septicum* by toxin-antitoxin reactions.

Another organism very close to *C. oedematiens* is the *Bacillus oedematis maligni* II of Novy (now called *Clostridium novyi*). It is often considered synonymous, but Weinberg regards it as an atoxic variety of his organism, remarking that it would probably have been so recognized had it been discovered *after*, instead of *before*, *C. oedematiens* (358).

*Bacillus gigas* Zeissler and Rassfeld (380) is also similar to *C. oedematiens*. It was found in a disease of sheep, resembling brad-sot or braxy, and was considered different from *C. oedematiens* principally because of the conspicuous size of its cells (1 to 2 by 4 to 20 micra) and because of certain cultural differences. It produces a toxin, whose relation to the toxin of *C. oedematiens* has apparently not been explored. Kraneveld, who found *B. gigas* in diseased animals in the Dutch East Indies, also reported

another organism (172), the bacillus of *osteomyelitis bacillosa bubalorum* of the Indian water buffalo; he later identified his organism with the "Novy group" as defined by Miessner, Meyn, and Schoop (173, 227). None of the descriptions of *B. gigas* is as complete as one would wish, and further study of its toxin is needed. The same may be said of other oedematiens-like organisms, which have been found in animal diseases: bradsot and braxy, infectious necrotic hepatitis, black disease, and big head of rams. These and probably others of the so-called braxy group are not as yet well characterized (with a few notable exceptions, e.g., black disease (243, 328)). Perhaps in time a complexity of causal organisms like that of the *C. welchii* group will be found, and if so, it is not improbable that knowledge of the toxin specificities of the group will aid in its resolution.

#### *The toxin of Clostridium chauvoei*

*Clostridium chauvoei* should perhaps be considered above, close to *C. septicum*, for it is of that general affinity. We choose to discuss it here, however, because for a time there was debate as to whether it formed a toxin, and certainly there are strains which have not been shown to do so in the laboratory. It thus represents, in a sense, a transitional organism to the non-toxigenic forms, and will serve to introduce mention of "non-toxic or atoxic" strains of supposedly toxigenic species.

*C. chauvoei* is the cause of blackleg, symptomatic anthrax, quarter evil, or "Rauschbrand," as the disease is called in different localities. Production of toxin was claimed by several early workers (151, 159, 171, 179, 236, 238) but denied by Scott and others as late as 1928 (130, 283-285). Recent work, however, has shown that toxin can be elaborated under proper conditions of culture (165, 214). The explanation of the disagreement seems to be that toxigenicity is easily lost, and consequently non-toxic strains are more generally encountered in this species than in other common anaerobes (8, 66, 130, 151, 167, 179). Perhaps because of the instability of the toxigenicity, more than usual effort has been made to practice active immunization against this disease. Many types of blackleg prophylactic products have

been tried: filtrates of cultures (at times probably toxin-containing), vaccines of various kinds, anacultures, even infected muscle powder and spore vaccines. These products may carry also lysins; and the immunity which they induce is undoubtedly complex. Hence it is very difficult to assess either the importance of the antitoxic factor in the immunity produced, or to claim for it any conspicuous laboratory usefulness in diagnosis or typing. As will be seen below, agglutination, precipitation, and complement fixation may be resorted to in the classification of such organisms.

### *The problem of atoxic strains*

It must be emphasized in passing that *C. chauvoei* is not unique in possessing atoxic strains. The literature cites the following: *C. tetani* (53, 84-86, 89, 163, 164, 206, 262, 263, 326); *C. welchii* (34, 35, 144, 183, 199, 201, 239, 240, 262, 289, 291, 336); *C. botulinum* and *C. parabotulinum* (17, 18, 45, 76, 113, 216, 241, 248, 267, 268, 322, 334); *C. oedematiens* (23, 39, 48, 359); *C. hemolyticum* (332); and the bacillus II of Ghon and Sachs (314). In most of the above cases the atoxic strains were found at isolation, or they developed spontaneously during laboratory cultivation of the originally toxic cultures. Occasionally, cultivation to produce variants was intentionally undertaken.

Loss of toxigenicity often throws a variant extremely close to the borders of another species. Who is to say that an atoxic strain of *C. tetani* is not within the confines of *Clostridium tetanomorphum*, or an atoxic *C. welchii* within those of *C. butyricum*? This weakness of the method, or perhaps one should say of our conceptions of the species, can in some cases be corrected by correlation of other antigenic reactions, agglutination or precipitation or complement fixation. Reference will be made to the placing of atoxic strains of *C. tetani* and *C. parabotulinum* by such means. But at least the possibility of missing entirely the relations of a strain, because of its atoxicity, brings the realization that the toxin neutralization test is not even a generally applicable method for typing anaerobes, since the many non-toxic species are entirely beyond its reach. In general applica-

tion therefore, any method of analysis of the intact cellular antigens is to be preferred. In our opinion, agglutination, properly carried out, serves that purpose, and its usefulness will be discussed next.

#### THE AGGLUTINATION REACTION APPLIED TO SPORE-FORMING ANAEROBES

First recognized during a comparison of the blood of animals normal and immune to tetanus, agglutinins for anaerobes were found soon after the phenomenon of bacterial agglutination itself was known (56, 57, 273). Early application was made to the distinction of two anaerobic species, *C. chauvoei* and *C. septicum*, by Leclainche and Vallée (178). Since then the method has had extensive use, as we can testify, having read more than 250 papers on this one phase of the serology of anaerobes! There has not been complete satisfaction with the method; and it will perhaps best serve the present purpose to mention some of its difficulties and failures, as well as its usefulness.

The first hint of difficulty appeared in a paper by Nicolle and Trenel (237) on the variability of agglutinative aptitude and agglutinogenic function, in which it was stated that anaerobes are: "infiniment moins sensibles et. . . souvent variables." Nevertheless, in the monographs on anaerobes after the World War (52, 75, 350, 378, 379) it was possible to list those organisms for which satisfactory agglutinating sera had been prepared, and the few for which they had not.

Although Meyer (200) had in 1915 recommended agglutination, Zeissler (376), Heller (135), and Hall (119) categorically condemned it as an aid in the typing of anaerobes. Hall and Stark (128) from their experience with *C. sporogenes*, however, stated that "one should regard serological agglutination as a satisfactory criterion of species identity in properly controlled positive tests, but not of differentiation in negative tests." One of the chief complaints concerned the instability or "auto-agglutinability" of the anaerobic cell suspensions used as antigens; for instance, "L'auto-agglutination des cultures du *B. oedematiens* en milieux liquides est une des caractéristiques de cet anaérobie. Il est

donc impossible d'étudier l'agglutination de ce germe en recourant aux procédés ordinaires." (345). It is unfortunate that the *Manual of Methods for Pure Culture Study of Bacteria* (41, 121) still recommends the use of broth cultures of anaerobes directly as antigen, either for injection or for macroscopic tube agglutination. Particularly in glucose broth, and some form of it is likely to be used with anaerobes, the acidity resulting from fermentation may cause flocculation of the cells. Heller (135) had the key to correction of this difficulty, when she secured stability by adjustment of the electrolyte concentration and the pH of the antigen suspensions. Snyder (294) recently re-emphasized the danger of non-specific agglutination by acid, and established a critical pH for the species with which he worked. If broth cultures are ever to be considered as antigens, it is important to know that the cells in broth may be flocculated at a pH distinctly higher than in distilled water. To avoid the errors due to auto-agglutination, Plaut (249) devised a dark-field method for agglutination in hanging drops; he claims that true agglutination is easily detected by the loss of motility of the clumps.

Two other complaints, (a) that for certain groups of anaerobes it is very difficult or "impossible" to produce agglutinating sera, and (b) that for others the agglutination may be ultra-specific, can best be dealt with by presenting the pertinent data for the major groups of anaerobes.

But first we would point out that methods of agglutination have undergone considerable change from the first simple mixing of culture and serum. The Oxford method of achieving "standard agglutination" was a distinct advance. It was followed by the flagellar-somatic agglutination of motile organisms, evidence for at least a dual nature of the agglutinogens. This concept was shrewdly criticized by Tulloch (327), as it was then a purely qualitative theory, little more than the old mosaic hypothesis of Durham and Ehrlich. But there can now be no doubt that separate antigens do exist independently within the cell, as evidenced by the many isolations of protein and polysaccharide fractions. Furthermore, the apparent success in resolving the *Salmonella* group by analysis of their somatic and flagellar anti-

gens is an outstanding example. The "Salmonella Subcommittee of the Nomenclature Committee of the International Society of Microbiology" (279) has recently published its findings on the genus *Salmonella*, in a scheme based on the Kaufmann-White antigenic factors of the group. It is of interest also that the League of Nations Health Organization has in April, 1937, adopted a detailed technique to be used in the agglutinative diagnosis of enteric infections (82). These recent uses, and a review and interpretation of the techniques of agglutination by Cruickshank (58), are commended to the reader. For specific agglutination methods which have succeeded with anaerobes, the following papers may be consulted: 83, 109, 136, 190, 281.

#### *The agglutination of Clostridium tetani*

As mentioned above, *C. tetani* was the first of the anaerobes to reveal its agglutinative powers. However, nothing of importance concerning the typing of the species appeared until Tulloch (324-326) discovered a subdivision into five serologic types. On a combination of the evidence from several workers (14, 50, 51, 85) nine types are now known. In 1928, the flagellar-somatic method of agglutination was applied to *C. tetani* by Felix and Robertson (83), and the dual nature of the agglutinogens was found for the first time to be true for a motile anaerobe as well as for motile aerobes. The type-specificity of former groupings (only seven were then known) was shown to lie within the reactions of the flagellar or thermolabile H antigen. There was included in the study a so-called "pure O" strain,—a non-motile variant by chance devoid of the H antigen proper to its species. Parenthetically, it may be said that such strains, if available, are used to produce potent somatic antisera; otherwise, the H antigen of a motile strain may be destroyed by heat or chemicals and the resulting product used as the O antigen. Antiserum for the pure O strain of *C. tetani* was found to agglutinate in the "small flaking manner"; and from this it was concluded that "in the stable antigen the group relationship is very close indeed," in contrast to the type-specificity of the H antigen. With so-called "smooth" and "rough" strains of *C. tetani*, Condrea (53)



soon confirmed the existence of H and O antigens in the species. His smooth strains were complete with both antigens, whereas the rough possessed only the O. The O antigen of any given rough strain, however, was like that of the smooth strains of its group. In other words, overlapping or very close relationship of O antigens between the groups was not confirmed. In fact, Con-drea stated clearly that in his opinion such cross-reactions must be due to admixture of types! There the matter rested until Gunnison (109) recently presented a thorough study of 67 strains, representing all nine of the types now known. As a result of the cross-testing of all strains with the antisera for all nine groups, "there was no differentiation among the 67 strains tested." Absorption tests being confirmatory, it was concluded that there is a common O antigen with no type specificity. But in addition to this common O antigen, absorption tests revealed another O antigen, which divides the collection into two sub-groups. Types II, IV, V, and IX possess this second O antigen, whereas I, III, VI, VII, and VIII lack it. The complexity of agglutination of *C. tetani* is therefore still considerable, but it has been clarified; and it should now be possible to identify an unknown strain as belonging to the species, if it possesses the common O or species-specific factor. *Clostridium tetanomorphum* was tested by Gunnison and, although it gave a group reaction to 1:640 titre, absorption with it did not lower the original O titre of the serum for *C. tetani*, thereby proving that *C. tetanomorphum* has not the species-specific O factor of *C. tetani*. *Clostridium tertium*, *Clostridium putrificum*, and *Clostridium sphenoides* were found to be even further removed in group relation. Among the 67 strains of *C. tetani* tested 11 were atoxic and therefore could not have been recognized as belonging to the species by a toxin-antitoxin test.

#### *The agglutination of Clostridium septicum*

Some early studies on the agglutination of *C. septicum* were made to distinguish it from *C. chauvoei* (178, 194, 283), apparently with success; although recently "a close relation" between the two organisms has been indicated by a combination of evi-

dence from their agglutination and complement fixation reactions (349). The complexity of its agglutination first became apparent in the report by Robertson (264) which indicated three subtypes in the species. Later, when flagellar-somatic agglutination was being applied to the species by Felix and Robertson (83), the same sera were reinvestigated, although nearly nine years old. It was found that the original subdivision depended upon the H antigen reactions, in this species as in *C. tetani*. Four groups were finally established by Felix and Robertson. In the somatic sera, they found the cross-reaction of the O antigens of the several groups to indicate close group relationship, but not identity. In 1928 Davesne (71) mentioned six subgroups in direct agglutination and therefore presumably dependent upon H antigens, but Bengtson (19) in 1933 still recognized only "at least four" groups.

The somatic or O antigen of *C. septicum* has an added importance in relation to protective immune sera, and has therefore been investigated by several workers. Robertson and Felix (266) claimed definite, but type-specific, protective value of the O immune serum. Weinberg, Davesne and Haber (348) were unable to confirm its value, and since antitoxic sera were successful, they saw no reason to change therapeutic procedure. Henderson, (139, 140), continuing investigation of the O antigen both for its species relationship and for its possible prophylactic value, confirmed the Robertson-Felix report. He did, however, clarify their statement concerning the close relation of the O antigens of the several groups. There is an identical O antigen in Groups II and IV; and "considerable overlapping," but not identity, in I and III. In conclusion, he suggested that the O antigen relationship be made the primary basis for grouping within the species, and that the H antigen relations be considered secondary. This suggestion has not been widely recognized, but it is, in fact, a good one, as the O antigen is the more stable. Henderson's most recent contribution (141) suggests that in addition to the O antigen, there is a heat-labile antigen, possibly identical with the H antigen, which has importance in anti-bacterial sera.

*The agglutination of Clostridium chauvoei*

After the early separation of *C. chauvoei* from *C. septicum*, the agglutination of *C. chauvoei* itself was examined (156, 226, 377, 378, 381). Strong reactions with "no serological differentiation of races" were found to occur, regardless of the ovine or bovine origin of the strains. Nevertheless, controversy as to the identity of so-called "spontaneous Rauschbrand" of sheep *versus* the "wound Rauschbrand" of cattle (209, 225, 226, 370) was not finally settled until a full antigenic analysis of the species by flagellar-somatic agglutination was accomplished. On this basis the H antigen was found by Roberts (260) to be different in the ovine and bovine strains, whereas the O was identical. Henderson (136), repeating the study, agreed upon the uniformity of the O factor; in fact, he claimed exceptional importance for it in view of the subdivision of species by means of the O factor in the case of *C. septicum*. He did not, however, confirm the separate entity of the ovine and bovine H factors. Instead he found an equal distribution of the H antigen in all but two of his strains (both English ovine which had a common H factor different from that of the other strains). A minor component of the H factor is shared by all strains. Later work deals only with the immunizing value of the O antigen in vaccine (137, 261) or in antibacterial serum (138).

*The agglutination of Clostridium parbotulinum and Clostridium botulinum*

The first investigators to study extensively the agglutination of *C. parbotulinum* (then called *B. botulinus*) were Starin and Dack (305) and Schoenholz and Meyer (281). The latter, examining 111 strains of diverse origins, found that the Type A strains could be divided into three or four subgroups and the Type B strains into at least two subgroups by agglutination and absorption tests. In each toxin type one group was left a heterogeneous collection of ultra-specific and unclassified strains. Only between certain groups was cross-agglutination noticed. Generally, negative results were obtained on cross-tests of the botulinus and the non-toxin-producing anaerobes, such as *C. sporogenes* and *C. bifermentans*.

Using the flagellar-somatic agglutination technique in an extensive re-study of over 160 cultures of the proteolytic *C. paratubulinum*, including the original collection of Schoenholz and Meyer, McClung (186) showed that the subgroups reported by previous workers were based on the reaction of the heat-labile antigens. Without exception, strains of *C. paratubulinum* Types A and B reacted with a serum produced against the heat-treated antigen of any strain regardless of toxin type. Mirror absorption experiments confirmed the identity of the heat-stable somatic antigen. Townsend (322) and Gunnison and Meyer (113) had previously classified various non-toxic strains by their reaction to titre with specific antisera; these strains also reacted with the somatic antisera and could not be distinguished from the toxic cultures. Even more interesting is the possibility of using a somatic antiserum as a specific reagent for the proteolytic *C. paratubulinum*, as revealed by the cross-tests of these organisms and various related species, in particular *C. sporogenes*. Although some cross-reaction is evident prior to absorption, it appears that somatic antiserum for *C. paratubulinum*, pre-absorbed by *C. sporogenes*, may give a reaction with the homologous species only. However, the strains of *C. sporogenes* do not all react uniformly; therefore a decision as to the group relationship of this species to *C. paratubulinum* must await further examination. Nevertheless, the great importance of such a serum is evident, and it is hoped that the future will soon produce a fuller study.

The heat-stable antigens of a small number of strains of Types B, C and D of *C. botulinum* were included in McClung's study (186), and their apparent specificity was noted. Prior to this, the agglutination of Types C and D had been examined by several workers (110, 224, 248, 268), and they too had found no cross-reaction with the type sera of *C. paratubulinum*.

#### *The agglutination of Clostridium welchii*

It is not yet possible to present the agglutination of *C. welchii* in anything like the state of agreement that has been reached for *C. tetani* and *C. paratubulinum*. There is nothing but confusion at present, and apparently for two reasons. As late as 1929,

Robertson (265) wrote that "there is no evidence that agglutinins have been produced in the bodies of animals injected with *B. welchii*." It is true that the literature contains rather numerous statements that such agglutinins are not, or at least not readily, produced. That they can be formed, however, is shown in some of the early studies. Several recent reports (149, 259, 344, 367, 375) of even comparatively high titres have removed all doubt on this score. However, the second difficulty, namely, that the antisera are conspicuously strain-specific, has not been overcome. We can do no more than quote Wilsdon (367): "While it is possible to prepare agglutinating sera in the case of a number of strains of *B. welchii*, there is little likelihood of formulating a satisfactory classification of the members of that group on the basis of their agglutination reactions." It is only fair to point out, however, that he refers to the entire *C. welchii* group, human and animal. It must also be pointed out that *C. welchii* has not been fully analyzed for antigenic structure by the newer techniques. The organism is non-motile, an exception to most of the group of spore-forming anaerobes. For this reason it has no flagellar antigen; but it does have capsular antigens to be considered. The precipitin and complement fixation reactions, which incidentally have not been adequately tried for the species, should offer possibilities.

#### *The agglutination of some proteolytic species*

It is convenient to consider together the agglutination of a group of non-pathogenic proteolytic anaerobes. There is as yet no certainty of a subdivision into types within these species, but there is interrelation of certain species, as recently revealed by agglutination studies.

*Clostridium sporogenes* is probably poorly defined, in the sense that it is considered a widely distributed species and that non-pathogenic proteolytic forms are often assigned to it without detailed study. Perhaps then confusion is to be expected. The several reports (75, 128, 200, 246, 378, 379) are not in agreement, except upon the fact that not all strains of *C. sporogenes* agglutinate in all antisera. Three groups were recognized by Zeissler and

Rassfeld (379), but Hall and Stark (128) claimed agglutination, if only to low titre, with all strains. Lack of homogeneity is again indicated in the two recent reports upon cross-agglutination of *C. sporogenes* and certain other proteolytic species. McClung (186) noted irregularity of behavior of certain strains of *C. sporogenes* in absorption of the antisera for *C. parabotulinum*, as discussed above. Another example of the interrelation of "certain strains" of *C. sporogenes* with another anaerobic species is reported by Smith (292). Stable rough variants of *C. histolyticum* are said to react, though not to full titre, in an antiserum for *C. sporogenes* (strain 319 only); a reciprocal reaction was also obtained with this strain. Other strains of *C. sporogenes* reacted only to 1:20 or 1:80 dilution of antisera for the rough variants of *C. histolyticum*. These findings were offered in support of a claim (147) that rough variants of *C. histolyticum*, which resemble *C. sporogenes* in colony form and certain biochemical properties, are not chance contaminants but show "genetic relation" to *C. sporogenes*. Smith, however, does not claim direct identity of the species, but merely "a number of antigens common to both *Clostridium sporogenes* and *Clostridium histolyticum*." Further study will be necessary to establish these relations.

*Clostridium bifermentans* has been studied recently in order to clarify its relation to the so-called *Bacillus centrosporogenes*. Since one of the principal differences claimed was the absence of motility in *C. bifermentans* and its presence in *B. centrosporogenes*, a study of the flagellar and somatic antigens seemed indicated. Proof of the complete identity of the antigens, coupled with the previously known identity of other characters makes it no longer necessary to recognize two species. Because *C. bifermentans* has priority, its name has been retained (47, 192). The merging of another species, *C. sordellii*, with *C. bifermentans* has recently been proposed, partly on the basis of cross-agglutination. Details of this proposal by Stewart (310) have been discussed under the section on the toxin of *C. oedematoides* (syn. *C. sordellii*).

Several other putrefactive species have been studied in recent years by Hall and his associates, and in each case agglutination has aided in establishing the species: *Bacillus paraputrificus*

Bienstock and *Bacillus capitovalis* Snyder and Hall (295, 297); *Bacillus difficilis* Hall and O'Toole, in which subgroups were recognized by Snyder (296); *Clostridium fallax* and *Clostridium carnis* (77); *Bacillus bifermentans* as separate from several others (47); and *Bacillus paraputrificus* Bienstock and "*Bacillus in-nutritus*" Kleinschmidt (124).

*The agglutination of the saccharolytic anaerobes of the butyric group*

Butyric anaerobes of the soil are a heterogeneous lot, and no one knows how many species to recognize! Serology has not given a complete answer by any means, but it has, we believe, contributed to the definition of several species (188, 190, 191). One such is *Clostridium acetobutylicum*, the organism of the original Weizmann method of butyl alcohol fermentation. An unusually complete set of cultures was available for the serological study of the species: two representatives of the original Weizmann isolation (one untouched for 17 years) and twenty other cultures isolated by various workers over a period of 20 years. A complete antigenic analysis was made by the H and O technique by both direct agglutination and absorption (190). Unquestionably, every strain belonged to the species, and there was no need to subdivide the group into types on the basis of either flagellar or somatic agglutination. The only variation encountered was quantitative, in the sense of Schütze (282); namely, certain strains were *master strains* with full complement of H and O factors, and others were *substrains* with the same antigens, but with some deficiency of the H factor. Since the latter is the flagellar antigen, it is not surprising that quantitative variation should occur from time to time.

In the group reaction of *C. acetobutylicum* with other butyric anaerobes there is nearest affinity to the retting organism, *Clostridium felsineum* Carbone, and to a new pigmented anaerobe which appeared different and which was later proposed as a new species, *Clostridium roseum* McCoy and McClung (191). The interrelations of the three species were analyzed with the following results (188). A common somatic O factor occurs in the three organisms, but each is distinguished by an H factor, which

is dominant and species-specific. The group reaction in the *C. felsineum*: *C. roseum* and *C. felsineum*: *C. acetobutylicum* crosses is accounted for entirely by the common O factor. That between *C. acetobutylicum* and *C. roseum* is due to the O factor plus some admixture of H factors, the *C. acetobutylicum* containing a minor fraction of the H which is dominant in *C. roseum*. Thus, serological evidence confirmed in another instance the division of species proposed on morphological and physiological grounds.

Another butyric anaerobe, *Clostridium thermosaccharolyticum* McClung (185), was analyzed by the same technique (187). It also is homogeneous, with variation only in the quantity of H factor in certain strains. The extension of flagellar-somatic agglutination to this species is of particular interest, because, as a thermophile, the organism grows at a temperature which would destroy the H antigen of a mesophilic species. Yet relatively, the H factor of the thermophile is thermolabile and comparable to the same factor in other motile species (189).

#### THE APPLICATION OF THE PRECIPITIN AND COMPLEMENT FIXATION REACTIONS TO ANAEROBES

##### *The precipitin reaction*

The close analogy between the precipitin and agglutinin reactions is well-known. Although much less antigenic analysis has been done by precipitation, there is a good agreement of results obtained thus far by the two methods as applied to the spore-forming anaerobes. Detection of precipitins in the diagnosis of diseases has been attempted (91, 95, 133, 247, 270, 372), and precipitating sera for a number of the species of anaerobes have been successfully prepared (30, 40, 81, 108, 116, 351). Precipitins can often be demonstrated with antisera prepared for general antimicrobial use (302). Weinberg and Barotte (344) have claimed that precipitins and agglutinins act together in a serum to give stronger reactions ("synergism of antibodies") without loss of specificity.

Probably the most extensive work on precipitation for the typing of an anaerobic species has been done with *C. paratubulinum* by Gunnison and Schoenholz (116). A large collection of Type A



and B strains was tested in precipitating antisera produced with washed and heated cells, and good agreement with the grouping by agglutination was found. Later (108), antisera were produced with the bacterial cell extracts obtained by the freezing-thawing technique. Such extracts contained both protein and carbohydrate constituents, but they were not antigenic *in vivo*; they were, however, specifically precipitable by antisera produced against the intact organisms.

Comparatively little is known of the specific carbohydrate constituents of the anaerobes. Jimenez (155) reported simply that such a fraction had been isolated from *C. welchii*, and Meisel (218) reported similarly for a *Bacillus amylobacter*.

#### *The complement fixation reaction*

Much the same success can be claimed for typing by complement fixation as by agglutination and precipitation, but the method has been even less extensively used. It has had its most considerable trial with the botulinus organism (115, 306,) and the tetanus organism (83). Highly specific reactions with groupings closely following those revealed by agglutination have been found. Gunnison and Schoenholz (115) pointed out one advantage: namely, that certain so-called inagglutinable strains can be assigned to groups by complement fixation.

Mention might also be made of an incidental use of the complement fixation reaction for detecting the botulinus organism or its toxin in spoiled canned foods (160, 306). It is also possible to determine with a high degree of accuracy the toxin content of a culture (308).

This brief presentation of the precipitation and complement fixation phenomena of the anaerobes is not in proportion to the importance of these reactions. But it cannot be claimed that either of them has contributed much to the typing of anaerobes, except to confirm previously known groupings. Precipitin tests will doubtless contribute significantly, after the groundwork has been laid by studies of the specific soluble substances of these species.

## A CRITIQUE OF SEROLOGY AS AN AID IN THE TAXONOMY OF ANAEROBES

Every bacteriologist must be troubled at times by problems of taxonomy. He may not himself be concerned with the technique and terminology of classification, but he would like to have *agreement* and *stability* as quickly as possible. To be told that bacteria are perhaps not classifiable is of no help. To be told that they can be classified only when more is known is of no present help. Nor is this quite true now, for there are groups of bacteria *within* which classification (in the common sense) has already been achieved. It is chiefly when he tries to comprehend the whole system of bacteria that he must conclude that taxonomy has failed. Yet he would probably still hold with Lehmann and Neumann (180) "that it is always necessary to strive after such a system."

The known best way to work toward a general classification is to build up one after another of the systems for special groups; and in that endeavor serological classification has its place. One can hardly expect to take an unknown organism, and knowing nothing of its morphology and physiology, to discover its identity by toxin neutralization. It may perhaps, produce no toxin. But one can, given a spore-forming anaerobe from the pulpy kidney, of a sheep, discover the toxin type. And having done so, one would have classified it intelligibly for others working with the *C. welchii* group. *Agreement* would be reached.

As to the *stability* of serological classification, it is possible to find arguments, both favorable and unfavorable. The major objections are that variation "changes" the antigenic reactions and that serology reveals a needlessly complex subdivision of organisms (which is perhaps another way of saying that antigenic variation has occurred). If the antigenic variation is so random that types are forever elusive, the objection is valid. But experience shows this not to be so. The very fact that a group of botulinus cultures, involving hundreds of isolations, can be sorted into five toxin types is significant. So too is the statement of Powell (251): "Uniformity in the results of agglutina-

tion and agglutinin absorption tests upon groups of single-cell cultures of the diphtheria bacillus, having common origins, indicates considerable stability in the agglutinative reactions of this organism. It has not been possible to split any parent culture on the basis of the agglutinative reaction of pure-line strains derived from it."

We do not imply that serological analyses have solved all problems of classification within groups. Certainly that cannot be claimed for any single method of serology. Agglutination of *C. welchii*, for example, has failed for reasons as yet unknown. We are aware also that there is no convenience in the considerable subdivision of *C. tetani* by its H antigen reactions; but we submit that the discovery of a common and species-specific O factor is useful. That the O factor of *C. paratubulinum* unites a part of the botulinus group is probably also significant. So also is the possible protective power of the "O vaccines" of *C. chauvoei*. So is the sharing of O factors between species in the butyric group. In short, it seems that *somatic* factors are most promising objects for further study. It is apparent also that group reactions require attention. The sharing of minor somatic elements among certain species (or among groups within a species) may show phylogenetic relationships useful to know. And conversely, removal of those group factors by pre-absorption of antisera may yield useful reagents for species analyses. One such case among the anaerobes has been indicated in discussion of *C. paratubulinum* and *C. sporogenes*.

Needless to say, all serological work requires great care. Improvement in purity and standardization of toxins and antitoxins, and greater use of cross-protection experiments for establishing the components of nearly related crude toxins, are the keys to further progress. In agglutination work greater emphasis must be placed on *complete* analyses with mirror absorptions. And finally, with precipitation reactions done upon isolated and if possible chemically defined fractions of protein and polysaccharide, it may be possible to reach that "Substantive Classification," which P. Bruce White has visioned (365).

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# ACCESSORY GROWTH FACTORS FOR BACTERIA AND RELATED MICROORGANISMS

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A number of studies in bacterial nutrition have dealt with the so-called growth factors, accessory substances, vitamins, or growth activators,<sup>1</sup> substances which in small amount appear to play an important part in the development of certain bacteria

<sup>1</sup> The term hormone has also been used occasionally to designate these substances, but it does not seem to be valid on the basis of usage in mammalian and plant physiology.

and allied forms. The nature of these substances together with their possible function in the metabolic activity of various microorganisms has been a matter of conjecture for some time. Recently some interesting results have been secured which should lead to a much better understanding of the entire subject.

In the past some confusion has been caused by a rather indiscriminate application of similar terms to a variety of effects. In some instances the effect of an added substance has been merely that of stimulation, resulting in more abundant or more rapid development in an environment in which cell proliferation was already taking place. This stimulation has been due, at times, simply to adding more available food material to a "starvation" medium. In other cases, however, the added material has seemed to play a more important part in that its presence appeared to be necessary for development. At times very small amounts of added substance have permitted good growth in a medium in which the organism was not ordinarily able to multiply.

Substances which exert one or another of these effects have been found in a wide variety of extracts of plant and animal tissues. Through fractionation of these extracts attempts have been made to obtain the active components in pure form and to learn something of their chemical structure. The observations have been scattered over a rather wide field dealing with many miscellaneous sources of growth factors and with microorganisms of quite diverse groups. Before reviewing this material in more detail it seems best to consider briefly the basic ideas which have guided the investigational work.

It has usually been assumed that unknown chemical entities of organic nature were supplied by the added extract, and that these hypothetical substances produced the growth-promoting effect. Other interpretations have been advanced from time to time. These can be summarized as follows: (1) The added material may have supplied certain needed inorganic salts, particularly those of metals which act as catalysts; (2) the added material may have combined with and removed from activity

an excess of some constituent which was present originally in sufficient concentration to be toxic; or (3) it may have altered the physical characteristics of the medium so that cell proliferation became possible. Sole reliance on any of these explanations makes unnecessary the assumption of the existence of essential accessory substances of organic nature.

At the present time we appear to have reached a point from which a clearer idea of these effects may be gained. In a few instances it has been found possible to substitute, either fully or in part, known chemical compounds for the indefinite growth-promoting entities of tissue extracts. Eventually we should be able to abandon the use of such terms as V and X factors, L fractions, and others of like nature as their specific identities become established.

It seems best to review first the fractionation of tissue extracts and other sources, pointing out those instances where the active substance has been identified or where a knowledge of its properties has progressed to such a point that its nature could be surmised and a known compound substituted successfully for it. A consideration of the other explanations of growth-factor activity will follow. No attempt will be made to review the earlier work, other than that needed as a background for the present discussion, since former studies have been summarized by Knorr (56), Sergeant (142), Peskett (111) and Knight (52). Also, no attempt will be made to treat the subject of plant auxins. Various bacteria and other fungi are important in the production of substances, such as 3-indoleacetic acid, which exert a marked influence on plant cell elongation and multiplication. So far as the writers are aware, however, there is no instance on record as yet in which auxin-*a*, auxin-*b*, 3-indoleacetic acid or related compounds have proved to be essential for cell proliferation of bacteria. The production of these substances by bacteria and their effects on plants is outside the scope of this article and readers are referred to the recent publications of Boysen-Jensen (6), Schlenker and Rosenthal (128), Went and Thimann (164), Nicol (101) and others.

## EXPERIMENTAL PROCEDURES

In experimental work dealing with the growth accessory factors for microorganisms details of technique are quite important. Although these items have been mentioned from time to time in studies on bacterial metabolism, often they have been neglected, and so it seems desirable to emphasize again certain points.

*Sterilization* of the fractions to be tested for growth-factor activity presents a problem at the outset. The unknown substances in tissue extracts may be destroyed by heat, while resort to filtration may cause serious loss due to inactivation or adsorption. The checking of one method against the other is useful, though there is the possibility that some of the factors may be inactivated by both procedures.

*Basic medium for the tests.* In tests for growth accessory factors, the basic medium should be adequate in all other respects and the conditions of cultivation should approach as closely as possible those known to be most suitable for the organism in question. Unfortunately our knowledge of these requirements is notoriously inadequate. Concerning many microorganisms little is known of their actual needs with respect to various amino acids and other nitrogenous ingredients, the inorganic salts and their proper physiological balance, and other factors such as osmotic pressure, surface and interfacial tension, gaseous environment, redox potential and kindred conditions. Consequently our efforts are often seriously hampered at the start, and it is uncertain whether these important conditions are being satisfied. To avoid the uncertainties regarding amino acid requirements, some investigators have used hydrolyzed casein or gelatin as a basic medium. This procedure, however, is subject to the disadvantage of introducing impurities with the casein and gelatin, and also in that the medium is no longer of known composition.

*Size of inoculum.* Inoculation of the test medium with large numbers of cells may introduce appreciable amounts of growth factors, either from the cells themselves or from the previous culture medium. While this added material may be eliminated by several successive transplants in the new environment, in

general it would appear to be more desirable to start with smaller numbers of cells, thus affording at the outset a stricter test of the ability of the cells to multiply in the new medium.

*Storage of growth factors in cells.* If cultures are grown in a medium containing an excess of some required factor, they may possibly store sufficient quantities of the material to permit of further limited proliferation when transferred to a deficient medium. This could well lead to erroneous conclusions or at least make difficult the interpretation of results.

*Multiple growth requirements.* Tissue extracts contain many biologically active substances. Liver, for example, has been shown to contain a number of the accessory substances as well as amino acids. Therefore in attempting to isolate growth substances for microorganisms due consideration must be given the multiplicity of possible factors. This is well exemplified in the work on bios. It must be borne in mind too that amino acids frequently accompany accessory factors through the earlier stages of attempted separation. If one or more of these amino acids is essential for growth and if it is not supplied in the basic medium, its subsequent removal during chemical manipulation will give rise to a deficient environment and the organism under investigation will be unable to multiply for this reason rather than because of a lack of other needed substances.

*Methods of assay.* Of the several methods which have been proposed for the quantitative determination of the potency of growth-factor preparations, by far the most widely used is that of visual inspection of the culture tubes for turbidity. This method has a large error but is simple and rapid. The use of a cell and thermocouple for the determination of the density of the suspension probably increases the accuracy of this method (169). More precise results can be obtained by direct count but where a large number of determinations is to be made, the time required would be a serious disadvantage. Direct weighing of the mass of organisms formed has been employed. In the case of the mycelium of molds (16) weighing probably gives accurate results, but with bacteria and yeasts it is doubtful whether the method is much more accurate than visual inspection. Indirect



determination (92) of the mass of bacterial growth by means of its nitrogen content is probably more accurate than inspection, although subject to some rather serious errors due to the possibility that nitrogenous material from the medium might be included with the organisms, or if the organisms are washed too thoroughly nitrogen may be lost. Sternfeld, Wermuth and Saunders (148) attempted to follow growth by means of changes in conductivity, refractive index and other physical properties of the cultures, but the differences were too slight to be useful. Some workers (50, 144) have used the titer of acid formed by bacteria as an index of growth.

In the following sections discussion of the growth-promoting materials for bacteria and the related non-chlorophyll bearing fungi has been arranged under the different groups of microorganisms.

#### VARIOUS GROUPS OF THE BACTERIA

##### *The streptococci and allied coccus types*

The nutritive requirements of the pathogenic streptococci have always been quite obscure. These organisms practically without exception fail to develop in various amino acid synthetic media (40, 64, 67, 29) and attempts at separation of essential growth substances from meat infusions or other similar sources seem to have been attended by unusual difficulties. In a few instances the active material has been carried through several preliminary stages in the process of purification but little success has been attained beyond this point.

By adsorption with fuller's earth and charcoal, Freedman and Funk (37) obtained from beef infusion, autolyzed brewer's yeast and peptone, substances which showed growth-stimulating activity for hemolytic streptococci. Substances with a similar growth-stimulating effect were also found on hydrolysis of certain proteins, particularly casein, commercial gelatin, yeast protein and edestin (37). The evidence indicated that the active substances were not constituents of the protein molecule itself. Mueller (91) showed that wood charcoal removed from beef heart infusion some component needed for development of the

streptococcus. The infusion could be reactivated by addition of small quantities of peptone or acid hydrolysates of casein and edestin. The activating material was separated by precipitation with heavy metals into two fractions which exhibited activity only after mixing.

Whitehead (165) applied precipitation with phosphotungstic acid to a tryptic digest of casein. Substances necessary for growth of a hemolytic streptococcus were removed with the precipitate but were not effective in supporting growth unless small quantities of the filtrate were also added. A further separation was accomplished by extraction with butyl alcohol. Hosoya and Kuroya (47) reported that an alcoholic extract of rice bran supplied something needed by hemolytic streptococci and that this material accompanied vitamin B. McLeod and Wyon (85) attempted to determine the property of fresh blood and serum which promoted growth of pneumococci and meningococci. This property of serum could not be extracted by butanol, and digestion of serum with trypsin destroyed it. They believed the effect of serum was a phenomenon of the colloidal state. Recently Rane and Subbarow (114a) reported that a mixture of glutathione, thiochrome, flavin, nicotinic acid, betaine, glucosamine and a calcium-alcoholic precipitate of highly purified liver extract, in a deficient basal medium, provided almost optimum conditions for growth of the Dochez NY5 strain of hemolytic streptococcus. Omission of one or more of these factors decreased the amount of growth.

The saprophytic streptococci, particularly those of importance to the dairy industry, have also received some attention. Orla-Jensen, Otte, and Snog-Kjaer (108) found that the active material in skim milk could be removed by adsorption on charcoal or fuller's earth and elution with a methanol-pyridine solution. This growth-promoting activity appeared to be due to several factors, one of which could be replaced by riboflavin. Wood, Andersen and Werkman (175) reported that growth of *Streptococcus paracitrovorus* was improved by the addition of riboflavin.

Working with several representative streptococci, Hutner (50) found that at least one factor could be removed from depro-

teinized milk by adsorption with certain brands of fuller's earth. The growth-promoting activity thus removed could not be replaced by the addition of pure compounds such as thiamin, riboflavin, uracil or guanine. The finding with respect to riboflavin is contrary to that of Orla-Jensen. Rahn and Hegarty (114) noted that lactic acid production by centrifuged and washed cells of *Streptococcus lactis* was increased regularly by the addition of 0.002 per cent nicotinic acid. Small amounts of ascorbic acid stimulated injured or exhausted cells. Adenine, inositol and riboflavin produced no effect. In several of these studies lactic acid-producing streptococci have been used along with the lactobacilli. A further consideration of this work is presented in a later section dealing with the *Lactobacillus* group.

Knowledge of the growth-accessory factors for the streptococci has in general not progressed much beyond the stage of impure tissue extracts. Although there have been isolated reports of the effect of chemically pure compounds, their efficacy in promoting growth of the various types of streptococci is not established at present.

### *Staphylococcus*

On fractionation of meat extract, Hughes (49) obtained an "activator" for staphylococci. This was effective in promoting growth in Uschinsky's medium or in a casein digest medium, in which freshly isolated strains were incapable of multiplication. The active material was concentrated to a point where the addition of .0001 milligram to 5 cc. of casein digest supported ready development. It was heat stable at pH 7.0, soluble in water, alcohol and acetone, but insoluble in ether and benzene. It dialyzed through collodion membranes and disappeared on acid hydrolysis of the meat extract. An apparently similar material was obtained from yeast extract ("marmite") by Knight (51). Typical strains of *Staphylococcus aureus* grew readily upon the addition of small amounts of this material to a basal medium of hydrolyzed gelatin, amino acids and glucose.

Further studies of this fraction by Knight, Fildes and associates have been instrumental in throwing light on the real nature of the active materials and their work constitutes an important

contribution to our knowledge of essential nutritive substances for bacteria. A high-vacuum distillate containing the active growth factor was used for further analysis. Biological indications from other sources, together with chemical and spectrographic evidence of their own, suggested the testing of cozymase, nicotinic acid, nicotinamide, and thiamin as definite compounds to replace the unknown yeast factor. Later, in a study of the absorption spectrum of the high-vacuum distillate secured from yeast, Holiday (45) concluded that nicotinic acid was present in the free state in the yeast concentrate.

It was found that the factor was a complex and that one component of it could be replaced by nicotinic acid (or nicotinamide) and the other by thiamin (Knight, 53). These two substances were not effective when added singly, but when supplied together a ready development of *Staphylococcus aureus* was secured. On substituting a collection of amino acids for the gelatin hydrolysate previously used, the organism was then grown in a medium the constituents of which were chemical entities of known structure (Fildes et al., 36).

The small amounts of nicotinic acid or its amide and of thiamin which sufficed for development were quite striking (Knight, 53, 54). A concentration of the amide of  $6.6 \times 10^{-7}$  M (0.08 microgram per cubic centimeter of medium) supported maximum growth in 27 hours in the synthetic medium, while light but still detectable growth was secured in the presence of  $2.6 \times 10^{-8}$  M amide. The smallest amount of thiamin which supported maximum development was about  $1.0 \times 10^{-8}$  M while  $5.0 \times 10^{-10}$  M produced a detectable effect. These amounts are equivalent to 0.003 and 0.00015 microgram per cubic centimeter of medium, respectively.

The activity of compounds related to thiamin was also studied by Knight (54, 55a). The components of the thiamin molecule, namely the pyrimidine plus the thiazole, were effective in place of the complete molecule (in the presence of appropriate amounts of nicotinamide). However, a number of other closely related compounds could not be substituted, indicating a high degree of specificity in the requirement of this organism.

For anaerobic growth of the staphylococcus, Richardson (121)

reported that uracil was required, a concentration of  $m/20,000$  being most effective. Twenty-one other related compounds were studied but showed no comparable effect. This striking specificity of uracil, it was suggested, indicates that the compound must be widely distributed in nature and that it exerts an effect which cannot be reproduced by adenine and its derivatives.

Another item concerning growth of the staphylococcus has been added by van Wagtendonk (cited by Kögl, 57). The addition of Kögl's "biotin" in the form of its methyl ester resulted in more luxuriant growth. Amounts of 0.005 and 0.05 microgram of the biotin ester produced a three- to four-fold stimulation of growth when added along with small amounts of thiamin and nicotinic acid. Biotin ester alone gave a slight increase in growth. In this instance a compound has been added which has been obtained in crystalline form although the chemical structure is unknown.

These results indicate that for best growth of at least some strains of staphylococci, something more than thiamin and nicotinic acid is needed. Since Knight reported very good growth of his *Staphylococcus aureus* in approximately 24 hours, it is possible that some strains may not need biotin or are able to synthesize it themselves. In a recent confirmation of Knight's work, the writers and associates (63) found that a strain of *Staphylococcus albus* developed in a synthetic medium containing thiamin and nicotinic acid, but growth was considerably slower than that secured in broth or after the addition of fractions of a spleen preparation to the synthetic medium. Evidently something else was needed by this strain for optimum growth, though whether this need could be filled by biotin is not known.

Whether or not another substance is needed for best growth of the staphylococcus, it has been demonstrated by Knight that this organism, which formerly failed to grow in synthetic media, can now be grown successfully by the use of chemically definite compounds. It is of interest to note that of the growth factors required by the staphylococcus, the two for which the chemical structure is definitely known (thiamin and nicotinic acid) are also needed for the normal functioning of the mammalian organism.

*The diphtheria bacillus*

The nutritive requirements of this organism have been the subject of many studies. Most strains refuse to grow in synthetic media composed of the usual amino acids, salts and sugar. Evidently something else is needed for development. Separation and identification of these additional growth factors have been the objectives of an interesting series of reports by Mueller and associates. Starting with a suitable medium containing either meat extract or other extracts of fresh tissues, they attempted to separate the components needed for cell multiplication (98, 93). Most of the growth-promoting activity of liver preparations was found in an alcohol filtrate of an aqueous extract, and substances essential for growth could be removed from such a solution by adsorption with wood charcoal and later recovered from the charcoal by elution with acid alcohol (93).

Further studies of the liver eluate by Mueller showed that the active materials could be separated into two fractions by repeated extraction of acid solutions with ether. Both fractions were required for the full growth-stimulating effect (99). The ether-extractable substance from liver could be replaced by concentrates of urine (cow and horse). On further investigation of this source and the use of fractional distillation with the Rittenberg apparatus an active substance was obtained and identified as pimelic acid,  $C_8H_{10}(COOH)_2$  (94).

When added to a basic medium consisting of casein hydrolysate, cystine, glutamic acid, sodium lactate and inorganic salts together with the ether-insoluble liver fraction, either the isolated product or synthetic pimelic acid produced a two- to three-fold stimulation of growth. Quantitative determinations of bacterial nitrogen (92) showed that the stimulating effect of pimelic acid became evident at a concentration of about 0.005 microgram per cubic centimeter of medium and reached a maximum in the presence of five to ten times this amount (94). Other dibasic acids of the same series, from oxalic up to azelaic, exerted no growth-stimulating effect.

Following the identification of pimelic acid, attention was next turned to the ether-insoluble fraction of liver extract. This

material, after combined esterification and acetylation, was subjected to fractional distillation; and growth-promoting activity appeared in both the lowest-boiling and the highest-boiling fractions. Nicotinic acid was substituted for the low-boiling fraction and showed the same growth-promoting activity. The most striking effect of nicotinic acid was exerted in a concentration of about 1.0 microgram per cubic centimeter of medium while approximately ten times as much nicotinamide was required to produce a comparable effect (95).

The high-boiling fraction of the vacuum distillate remained as the only source of unidentified material and this was next subjected to examination by Mueller and Cohen (97). Chemical evidence indicated the presence of amino acids and it was found that  $\beta$ -alanine, which had been shown by Williams and Rohrman (171) to exert a growth-promoting effect on yeast, could be substituted for the high-boiling material.  $\beta$ -Alanine produced its maximal effect in a concentration of about 1 microgram per cubic centimeter of medium. *l*-Carnosine ( $\beta$ -alanyl histidine) was also effective but a greater concentration was required (96).

As a result of these studies it was shown that three substances of known chemical structure could be substituted for the hitherto unknown materials in extracts of liver and other tissues. In comparing the effect of these three compounds on the growth of four strains of diphtheria bacilli it was evident that each of them, when supplied alone in the basal casein-hydrolysate medium, exerted no appreciable growth-promoting effect.  $\beta$ -Alanine and nicotinic acid together were quite effective, and for some strains pimelic acid exerted an added stimulative effect (97). On substituting amino acid mixtures for the basal medium of hydrolyzed casein, several cultures of the Park 8 strain developed readily and produced a potent toxin in a medium of definite chemical composition (110). These reports by Mueller and associates provide an excellent example of the value of intensive and thorough search for the substances in tissue infusions which are required by some bacteria; and they constitute an important contribution to our knowledge of the nutritive requirements of bacteria.

The rôle of  $\beta$ -alanine and nicotinic acid was recently confirmed by other workers (63) though  $\beta$ -alanine appeared to be the more important of the two substances insofar as one Park 8 culture was concerned. Addition of  $\beta$ -alanine alone to a basal medium of amino acids, dextrose and mineral salts resulted in growth of a strain of the organism which failed to develop without the  $\beta$ -alanine. In contrast, nicotinic acid or pimelic acid alone did not support growth but the former stimulated development when added with  $\beta$ -alanine.

While Mueller's work has done much to clarify our knowledge of the usual requirements of this organism, it should be added that apparently some strains of diphtheria bacilli either do not require the foregoing compounds or else are able to synthesize them, for evidence has appeared from time to time that occasional strains of the organism may be cultivated in ordinary amino acid synthetic media and in this earlier work  $\beta$ -alanine and nicotinic acid were of course not used. Typical of such reports are those of Braun, Hofmeier and Mundel (7), of Maver (84) and of Wadsworth and Wheeler (162). The last mentioned investigators obtained growth of thirteen out of twenty recently isolated virulent strains in a synthetic medium which did not contain  $\beta$ -alanine and nicotinic acid. Development of the cultures was slow, but could be carried through successive transplants and weak toxin was produced.

### *Dysentery bacilli*

Many strains of dysentery bacilli fail to develop in the usual synthetic media composed of amino acids, glucose and inorganic salts; evidently other substances or conditions are required. Upon addition of small amounts of tissue extracts to such a medium, the cultures usually develop readily. The growth-promoting substances in veal infusion, yeast, and other animal and plant tissues can be obtained in impure form by charcoal adsorption (65). They can also be partially purified by treatment of tissue infusions with solutions of heavy metals which precipitate inert material, but do not precipitate the growth factors (127).

Recently it has been shown by Koser, Dorfman and Saunders



(61) that nicotinic acid or nicotinamide can be substituted for the fractions from tissue extracts and thus it is now possible to secure growth in a medium of definite chemical composition. Amounts of 0.1 microgram of nicotinic acid per cubic centimeter of synthetic medium caused prompt growth with pronounced turbidity of a number of Flexner and Sonne strains, while 0.01, 0.004 or at times even 0.002 microgram per cubic centimeter sufficed for slower and scantier development. Whether the growth-promoting property of the tissue extracts is due to nicotinic acid or to the amide is not known, since the presence of these compounds has not yet been definitely established in these preparations.

It is of interest that for the dysentery bacilli nicotinic acid, or its amide, seems to be the only substance needed in addition to amino acids, glucose and salts. In the case of the staphylococci, it will be recalled, both nicotinic acid (or the amide) and thiamin were needed, and for the diphtheria bacillus a combination of nicotinic acid and  $\beta$ -alanine gave the best results.

### *Brucella*

Growth-promoting substances in extracts of yeast and beef liver were precipitated with phosphotungstic acid and found to be remarkably stable on heating in the presence of acid or alkali (48). Koser and Saunders (65) found that growth-promoting activity for *Brucella* could be removed from various plant and animal tissue extracts by charcoal adsorption and recovered by subsequent elution with alcohol or acetone. The active substances could also be concentrated by precipitation of inert material with heavy metals. Substitution of various definite compounds for the active fractions of tissue extracts has not been successful. Thus, the addition of nicotinic acid, thiamin, riboflavin,  $\beta$ -alanine and other compounds to a synthetic medium was not followed by growth of a *Brucella abortus* culture (63).

Development of some *Brucella* cultures in synthetic media without the addition of added growth factors has been reported by ZoBell and Meyer (179), though even the most promising of their synthetic media were far from satisfactory. Growth was slow and some cultures refused to multiply in the second trans-

fer. Furthermore, from one hundred thousand to a million cells per cubic centimeter of synthetic medium were needed to insure positive results. The inability of small numbers of cells to initiate growth suggests that other factors or conditions were needed.

### *The hemophilic bacteria*

Studies of the accessory requirements of *Hemophilus influenzae* and allied hemophilic types have received more attention and are better known to most bacteriologists than those dealing with other groups of microorganisms. The reports of Davis (20, 21), Thjötta and Avery (153, 154, 155), Fildes (32) and others demonstrated that two substances were necessary for development of Pfeiffer's bacillus. One of these was associated with the hemoglobin of blood and the other occurred in a variety of plant and animal tissues or in the extracts of microorganisms. Extracts of potato apparently contained both substances. From these studies there emerged the now-familiar V and X factors. Separately these factors are not sufficient for cell proliferation of *H. influenzae* but when supplied together in ordinary culture media prompt development occurs.

The V factor is thermo-labile, diffuses through parchment membranes and is easily destroyed in alkaline solution. Its potency is lowered or it may be completely inactivated on contact with fresh serum. It is produced by a number of bacteria as well as by yeasts and molds and is found in many plant and animal tissues. The X factor is relatively thermo-stable and is associated with the iron-containing fraction of hemoglobin. It may be replaced by hematin. It occurs in plant tissue, especially potato, and is probably elaborated by some bacteria. It is often, though not always, associated with peroxidase activity and it has been suggested by several workers that its action is of a catalytic nature, accelerating the transfer of oxygen from peroxides in the medium or from the atmosphere to the bacillus. The essential points with respect to the V and X factors were confirmed and extended by a number of workers during the several years following 1921. No detailed account of these

results need be given here since this material has been covered in previous reviews (141, 111, 52).

The requirements of other organisms of the influenza group have been studied to some extent. Certain representatives of this group required only the V factor and were termed *H. parainfluenzae* by Rivers (123). Among these influenza-like bacilli there were encountered both hemolytic and non-hemolytic strains which possessed the common characteristic of being able to develop in the presence of the V but without the X factor (33, 160). In contrast to these organisms are the so-called *B. hemoglobophilus canis* of Friedberger which requires only the X factor (Rivers, 122) and Ducrey's bacillus of soft chancre (*Hemophilus ducreyi*), which according to Lwoff and Pirotsky (79) needs for its growth the X factor (hemin) but not the V factor.

More recently some additional information on the nature of the V factor has appeared. The earlier suggestions that the V factor might be vitamin C (ascorbic acid) appear to have been definitely ruled out by Meyer (86). Studies on the coenzyme of Warburg and the cozymase of Harden and Young have afforded the basis for an important step in our understanding of the V factor. Lwoff and Lwoff (76) found that either the coenzyme or the cozymase could be substituted for the unknown V factor and *H. parainfluenzae* then developed readily in peptone solution. It is interesting that nicotinic acid, its amide, diethylamide and adenylic acid could not be substituted for the coenzyme or V factor. This represents a contrast to the staphylococci and the dysentery bacilli which either are able to synthesize the codehydrogenase or can utilize the constituent parts as such.

It was found also (77) that the codehydrogenases had no influence upon the speed of reduction of methylene blue or of oxygen uptake by cells of *H. parainfluenzae* grown in the presence of an excess of V factor, but did increase these processes by cells grown in the presence of small amounts of V factor. When approaching the limit of active dilutions, the action of the codehydrogenases was quantitative. Evidently the physiological function of V factor is that of a catalyst in cell oxidations. Similar evidence was submitted by Lwoff and Lwoff (78) with respect to

the rôle of hemin (X factor). They believe that the function of hemin as a growth factor is in the formation of respiratory enzyme systems such as cytochrome, cytochromoxidase, catalase and peroxidase. These interesting contributions supply evidence concerning not only the chemical nature of the hitherto mysterious V factor, but also the rôle of both the V and X factors.

### *Acid-fast bacteria*

*Johne's bacillus* usually fails to develop even in the more complex media, unless killed cells or extracts of other acid-fast bacteria are added. Twort and Ingram (157) attempted to isolate the substance essential for *Johne's bacillus* from cells of other acid-fast types, such as *Mycobacterium phlei*. Several extracts were prepared and from one of them a small amount of the active substance was precipitated with barium salts. Further purification was not attained. The growth-promoting activity was not destroyed by autoclaving. While it is uncertain whether the so-called "essential substance" studied by Twort and Ingram over twenty-five years ago can be classed with the accessory factors, the work nevertheless possesses considerable interest as being one of the earliest attempts to isolate growth substances from complex mixtures.

*Tubercle bacillus*. Of the various pathogenic bacteria the tubercle bacillus is often regarded as one of the less exacting in its nutritive requirements, since many strains may be grown in the simpler synthetic media, of which Long's is probably the best known. There is evidence, however, that additional substances are required for maximum development and that the tubercle bacillus is unable to initiate growth in Long's medium unless the solution is seeded with large numbers of cells. Uyei (159) states that growth in Long's medium occurred only when the inoculum contains 1 milligram of cells (about five billion), whereas in Petroff's glycerol-egg medium and in a potato-glycerol medium development of cultures could be secured with inocula of 0.001 milligram and 0.000,000,001 milligram, respectively.

Addition of a yeast preparation or of orange, tomato or cabbage juice to Long's medium increased markedly the amount of growth

of both human and bovine types of the bacilli after 2 and 3 weeks of incubation (Uyei, 158). Of the several interpretations which may be made from such an observation, Uyei emphasized the supposed vitamin-like nature of the accelerating substances and suggested a relationship to vitamin B (complex). Uyei (159) also studied the nature of the growth-promoting principles of the potato and reported that the active substances could not be extracted with acetone, alcohol, or ether.

Not only is the nature of growth accessories for the acid-fast organisms unknown, but there is doubt as to whether such substances are required for the better-known representatives of the group.

*The anaerobic spore-formers: genus Clostridium*

A "vitamin" necessary for cell proliferation of *Clostridium sporogenes* was described by Knight and Fildes (55). Cultures of the anaerobe failed to develop in an acid hydrolysate of photographic gelatin supplemented by tryptophane, sodium citrate, thioglycollic acid and inorganic salts unless small amounts of the "sporogenes vitamin" were also added. Two-tenths of a microgram in 10 cc. of basal medium was sufficient for growth just visible to the eye. The active material was obtained in the form of a yellow gum from yeast and could also be obtained from human urine. The same substance, or something capable of replacing it, was also synthesized by certain microorganisms, notably *Salmonella aertrycke*, the tubercle bacillus, and *Aspergillus versicolor*.

Substitution of an amino acid mixture for the gelatin hydrolysate was made possible through the study of Fildes and Richardson (35) and development of *Cl. sporogenes* was then secured in a medium the only unknown component of which was the "vitamin." Fildes (34) has presented evidence to show that the "sporogenes vitamin" is also needed by many strains of *Cl. botulinum*. Pappenheimer (109) made a further study of the chemical properties of this growth factor. Highly active preparations were secured but the substance could not be obtained in crystalline form. The material had the properties of an unsaturated hy-

droxy-acid of molecular weight about 200, and the formula  $C_{11}H_{14}O_4$  or  $C_{11}H_{11}O_4$  was suggested. The factor was considered to be distinct from the plant auxins, Williams' pantothenic acid, the staphylococcus factor of Hughes, and the bios of Kögl.

*Propionic acid bacteria; lactobacilli; butyl alcohol bacteria*

*Propionic acid bacteria.* These microorganisms are usually considered to be fastidious in their growth requirements since they do not multiply readily in the ordinary peptone medium, but develop much more rapidly upon the addition of milk whey, yeast extracts or tissue extracts. Van Niel (161) believed the superior fermentation obtained in the presence of yeast extracts and yeast autolysates could not be ascribed to differences in nitrogen content or buffer capacity and suggested that accessory substances in yeast might play an important part.

The stimulative effect of potato extract, orange juice and yeast-water on glucose fermentation and acid production by these organisms was studied especially by Fromageot and Tatum (38) and by Tatum, Peterson and Fred (150). Evidence indicated that the stimulative activity of potato extract was not due primarily to available nitrogen content or buffering capacity. By use of the Neuberg reagent (mercuric acetate and sodium carbonate) the potato extract was separated into two fractions both of which were needed for maximum stimulation. The Neuberg filtrate fraction was believed to contain some accessory substance other than mineral salts, since after ignition the ash did not produce the complete stimulative effect (150). The effect of the Neuberg precipitate was due primarily to ammonium nitrogen and asparagine. Ammonium nitrogen was utilized in the presence of the proper growth factors (151).

In a continuation of this work, yeast extract was used as the source of growth stimulant and from it Wood, Tatum and Peterson (176) obtained a fraction apparently essential for growth of various strains of propionic acid bacteria in a glucose-ammonium sulphate medium. This factor was acidic in nature, non-volatile and could be extracted with ether. It could not be replaced by other biologically active substances, namely thiamin,

the flavin fraction from liver, the sporogenes vitamin of Knight, Williams' pantothenic acid, indoleacetic acid, inositol, or nicotinamide. It should be emphasized, however, that the propionic cultures did not grow indefinitely in the glucose-ammonium sulphate-yeast factor medium, indicating the need for some additional material. This was supplied by hydrolyzed casein or by unhydrolyzed casein, egg albumin or milk powder. From these sources, as well as from yeast extract, the active material could be extracted with alcohol and acetone. It was neither an amino acid nor a part of a protein molecule (Tatum, Wood and Peterson, 1952). The solubilities and stability of the active fraction resembled those of thiamin and this similarity suggested substitution of the pure vitamin. Two different samples of thiamin were found to be capable of completely replacing the extract. One sample was effective in amounts of 0.005 microgram per cubic centimeter of medium while 0.05 microgram of the other lot was required. Inositol, pantothenic acid, ascorbic acid, heptoflavin, nicotinamide and indoleacetic acid were not effective in replacing the extracted material. Here we have an interesting instance of the replacement of an unknown growth-stimulating material by a known substance of definite chemical composition, thus advancing materially our knowledge of the physiological requirements of the propionic acid bacteria. Evidently, at least one other substance is needed and it is contained in the acid-ether extract of yeast or potato.

There is also evidence that riboflavin is a stimulant for propionic acid bacteria. On fractionating yeast extract, Lava, Ross and Blanchard (69) found the B<sub>2</sub>-containing portion to be the most active in stimulating acid production. This was confirmed with pure riboflavin by Wood, Andersen and Werkman (175). They also found (175a) that the factor in the ether extract of yeast extract was essential for all cultures of propionic acid bacteria. This factor could not be replaced by a mixture of nicotinic acid, thiamin, pimelic acid, uracil,  $\beta$ -alanine and "pantothenic acid." Riboflavin and thiamin stimulated growth but were not essential.

*Lactobacilli.* Orla-Jensen, Otte, and Snog-Kjaer (108) stated

that riboflavin and one or more other "activators" are necessary for normal development of certain lactic acid bacteria. Their conclusion that one of these substances is pantothenic acid seems questionable, however. Their finding concerning riboflavin was confirmed (175). Other unknown substances were also needed and the requirements varied somewhat with different lactic acid types. Unknown factors in the basal medium (175) were the ether-soluble component from yeast and hydrolyzed casein. Seventeen purified amino acids did not satisfactorily replace the hydrolyzed casein.

Snell, Tatum and Peterson (145) reported that two unknown factors appeared to be necessary for attainment of luxuriant growth by *Lactobacillus delbrückii* in a hydrolyzed casein medium containing added tryptophane and a fermentable carbohydrate. One of these factors occurred in the Neuberg filtrate fraction or in an acid-ether extract of crude potato extract. The evidence suggested an acid of fairly low molecular weight. The second factor occurred in peptone, was basic and could be precipitated with Neuberg's reagent and with lead acetate and ammonia. Liver extract contained both of the growth stimulants, or other substances capable of replacing them.

In a later report Snell, Strong and Peterson (144) found one of the factors in liver to be an acidic, ether-extractable organic substance. The maximum effect of this fraction was attained in the presence of 0.1 to 0.3 microgram per cubic centimeter of basal medium, though its effect was detectable with amounts as small as 0.003 microgram. The basal medium contained riboflavin, which also exerted a stimulating effect in small amounts, and sodium acetate in addition to other more commonly used substances. A number of known compounds were tested but failed to replace the fraction from liver. These were: auxin- $\alpha$ , 3-indole-acetic acid, pimelic acid, pyruvic acid, uracil, and combinations of nicotinamide and thiamin. The relationship of this substance from liver to those previously described and to the ether-extractable substance for propionic acid bacteria is not clear at the present time.

*Butyl alcohol bacteria.* Recently Brown, Wood and Werkman



(9) obtained an acidic, ether-soluble fraction from yeast extract which was essential for vigorous growth of butyl alcohol organisms in a medium consisting of hydrolyzed casein, tryptophane, ammonium sulphate, glucose and inorganic salts. When a mixture of 18 purified amino acids was substituted for the hydrolyzed casein the organisms refused to grow. The hydrolyzed casein apparently contained a second unknown factor or else an essential amino acid in addition to those used. Here again, as in much of the previous work reviewed in this section, it appears that at least two substances are needed, one occurring in the acid-ether extract of yeast and the other in hydrolyzed casein. Werkman and associates (9) state that the latter is not thiamin.

Insofar as the requirements have been elucidated, it is evident that these fermentative bacteria, which are not associated with invasion of animal tissues, nevertheless require some of the vitamins which are essential for the higher animal.

### *Nitrogen-fixing bacteria*

Growth of the various types of *Rhizobium* in synthetic media is usually negligible if pure, ordinary ingredients are used. Upon the addition of small amounts of yeast extract the cultures develop readily and evidence has been advanced, notably by Allison and Hoover (1, 46), that the effect of the yeast can be attributed to the presence of small amounts of a growth factor. This substance they termed "coenzyme R." It was found to be present especially in yeast, cane molasses, natural humic acid, commercial egg albumin, and commercial sucrose. It could be obtained along with impurities by extraction of commercial sucrose or dried cane molasses with absolute alcohol. Small amounts of such extracts, when added to the usual synthetic medium, led to good development of cultures of the root-nodule organisms (1). The extracts also stimulated the rate of respiration as determined in the Warburg apparatus.

Hoover and Allison (46) obtained apparently the same factor in more concentrated form from *Azotobacter* cultures which had evidently synthesized it. Attempts to obtain it in crystalline form were not successful. The substance was dialyzable and

quite heat-stable. It was not identical with Williams' pantothenic acid. Cystine and related reducing substances, inositol, synthetic iron humates, and various nucleotides could not be substituted for it. They also report (2) that the growth response of the nodule bacteria to natural humic acid is due almost wholly to this factor and not to the available iron content. The presence of a somewhat similar substance in brown sugar and in calcium sucate has been noted by Clark (17). This substance was responsible for growth acceleration of *Rhizobium trifolii*. It was destroyed by ashing, was dialyzable, was adsorbed by charcoal and was reported to resemble in some respects the bios complex of yeast.

It is difficult to correlate these reports and at present no conclusion can be reached concerning the nature of any growth factor which might be needed by *Rhizobium* or other nitrogen-fixing soil organisms.

### *Streptothrix*

Attempts were made by Reader and associates to separate growth-promoting substances for *Streptothrix corallinus* from an enzymic digest of beef. No pure compound was obtained but the active material was stated (116) to be organic, water-soluble, ether-insoluble, dialyzable, stable to alkali in the purest preparations, and not precipitated by neutral or basic lead acetate. It was not identical with vitamin B<sub>1</sub> or B<sub>2</sub> preparations (112), but a similarity in constitution to B<sub>1</sub> was suggested. In later work (117) it was found that mannitol, but not the other alcohols commonly used in bacteriological work, considerably increased the mass of growth when added to the salt-sugar medium together with a growth factor preparation. It was believed that the mannitol acted as a specific source of food rather than as an additive growth-promoting factor, and a similar interpretation was suggested for the effect of *i*-inositol (bios I) upon yeast.

The relation of this streptothrix growth substance to the other bacterial growth-promoting factors is not clear. Repetition of the tests with pure preparations of thiamin and the other compounds now available would be of interest.

## YEASTS

A consideration of growth accessory substances for the yeasts revolves largely about the question of "bios," the term first applied in 1901 by Wildiers (166) to designate material in yeast extract which was needed for normal cell proliferation of *Saccharomyces cerevisiae* in a synthetic medium. Wildiers and a little later Devloo (22) recorded some of the physical and chemical properties of bios and stated that it was not present in yeast ash. The study of the accessory growth substances for microorganisms may be said to have started with this work.

It will be recalled that up to this time Pasteur's opinion had prevailed, namely that yeast could be cultivated readily in a solution consisting only of sugar, an ammonium salt and ash of yeast. To this Liebig had objected and offered evidence to the contrary, though the weight of opinion continued to favor Pasteur's view. Wildiers proposed a possible explanation for these differences based on size of the inoculum and consequent carrying over of bios. He evidently considered his term bios to be only a tentative one, expressing the hope that a chemical name might later replace it. In this he appears to have been years ahead of his time for a quarter of a century was to elapse before any real progress was made in this direction.

Wildiers' ideas were soon challenged and for a few years a controversy ensued concerning his interpretations and methods. This has been reviewed by Tanner (149) and Buchanan and Fulmer (10). The discussion of bios largely disappeared for a time, only to be revived some years later when it was maintained that vitamin B and bios were the same and that the stimulation of yeast growth by bios supplied a quantitative method of assaying vitamin B (168). It was soon shown that this assumption in its original form was incorrect. However, a renewed impetus was given and much of our present knowledge of bios is due to the intensive studies started at this time by several groups of investigators, especially those associated with Fulmer, Miller, and Williams.

Evidence shortly appeared to show that bios was in reality a complex of a number of substances and that the combination of

all of them was often necessary to exert the stimulating effect (72, 39, 173). Similar situations have been encountered more recently in the study of other growth factors. Along with recognition of the multiple nature of bios further complications arose when it was realized that different yeasts possessed quite different nutritive requirements. In the earlier work there had been a tendency to regard yeast as a single entity. Some of the contradictory statements found in the literature undoubtedly were due to this failure to recognize the marked differences between species and strains. This situation was remedied, however, as attention was directed to the differing nutritive requirements of various yeasts. Lucas (72) found that different strains of yeast varied in their response to bios, and similar findings were reported by Williams, Wilson and von der Abe (173), Copping (18) and others. In the more recent studies this difference in requirements has been well recognized.

Aside from the yeast cell itself, many other sources of bios-like growth stimulants have been reported. Wildiers (166) originally called attention to several of these sources, and more recently the presence of growth factors for yeast has been noted in alfalfa (39), the buds and leaves of a number of plants (19), oat coleoptiles (27), tomato juice (28, 88) and commercial sugars (43). All the evidence indicates a widespread distribution in nature.

Regarding the chemistry of the bios complex, the separation accomplished by Lucas (72) seems to have been the basis for much of the subsequent work. He obtained two fractions, bios I and bios II, by treating with alcoholic barium hydroxide. Separately each fraction possessed little activity but when combined the original activity was restored. In subsequent work by Eastcott (25) the active principle of bios I was identified as *D*-inositol.

Later work showed that bios II was not a single entity and several groups of investigators fractionated it by one method or another. Miller, Eastcott and Sparling (89) recognized a bios II A and bios II B. Crude bios II B contains a new constituent provisionally named bios VII (88). It was reported (87)

that II A could be replaced by  $\beta$ -alanine and leucine. Also a bios V which appeared to be necessary for a certain strain of yeast was reported by Farrel (28). The bios V, however, did not increase the crop of *Saccharomyces cerevisiae* or several other common yeasts. Bios V can apparently be replaced by thiamin (88).

In the meantime Williams and Roehm (170) found that thiamin stimulated growth of some yeasts and pointed out that it possessed certain properties in common with one of the components of bios II. In addition, there was evidence of marked growth-promoting activity in another fraction. In further work Williams and associates (169) reported the presence and partial purification of an acidic substance which markedly stimulated growth. This substance was of widespread occurrence in nature and was called "pantothenic acid." Small amounts of pantothenic acid alone, when added to a basal medium, exerted some growth-promoting effect, but the activity was enhanced by addition of *D*-inositol or thiamin or both (Williams and Saunders, 172). Richards (120) has reported that pantothenic acid stimulates yeast growth by shortening the generation time. This was seen particularly when the seed yeast came from older cultures. There was less effect on the crop.

Williams and Rohrman (171) added  $\beta$ -alanine to the list of growth-promoting compounds. When incorporated in a basal medium containing salts, sugar and inositol, 0.08 microgram per cubic centimeter of  $\beta$ -alanine produced growth stimulation of five yeast strains. The addition of aspartic acid to the medium resulted in a still larger yeast crop, while one of the five strains also required thiamin.

Recently Kögl and Tönnis (59) announced the isolation of a substance called "biotin" which was obtained in crystalline form as its methyl ester. This was isolated from what constituted part of the bios II complex (the fraction adsorbed on charcoal). Biotin possesses a marked stimulating effect on yeast growth, a dilution of one part in  $4 \times 10^{11}$  producing a perceptible effect, while one part in  $4 \times 10^{10}$  caused a more distinct stimulation.

In much of the foregoing work various strains of *Saccharomyces*

were used. Schopfer (134, 135) has recently studied the requirements of several of the torulae, *Rhodotorula rubra* and *R. flava*. These yeasts required thiamin for satisfactory development, and maximum growth was obtained with about 0.4 microgram in 25 cc. of synthetic medium. The pyrimidine component of thiamin could substitute for the whole molecule; the thiazole component was practically without effect. Inositol and pantothenic acid or combinations of both produced no growth-promoting effect upon these two species. Further differences in the requirements of different yeast strains with respect to thiamin and its two component ring structures have been reported by Schultz, Atkin and Frey (140).

In an interesting report Sperti, Loofbourow and Dwyer (146) have directed attention to the liberation by injured cells of substances affecting growth. Cells of *Saccharomyces cerevisiae* injured by ultraviolet irradiation produced substances which stimulated cell proliferation. Apparently the effect was due not merely to substances found in normal cells but to products elaborated by the injured, living, cells as a definite response to injury. These results are of interest in relation to similar evidence concerning the proliferation of cells in tissue cultures. Also they may have a general bearing upon the preservation of communities of microorganisms following injury to some of the cells of the community. Norris and Kreke (105) have presented evidence to show that the factors affecting growth, fermentation and respiration of *Saccharomyces cerevisiae* are not one and the same substance. Using malt combings as a source of bios, they showed that factors which affect these different cell activities could be concentrated in different fractions.

To sum up the evidence, it is quite apparent that the yeasts as a group vary as widely in their requirements of accessory growth substances as do the bacteria. Some yeasts can develop through continuous transplants in simple synthetic media and evidently are able to synthesize all needed compounds. Others are stimulated in greater or less degree by additions of *D*-inositol, thiamin,  $\beta$ -alanine, "biotin" and "pantothenic acid," depending upon their inability to obtain by synthetic or other processes one or more of

these materials. Doubtless still other compounds will be found to be needed by some of the more exacting species.

#### MOLDS AND HIGHER FUNGI (EUMYCETES)

Although many of the fungi develop readily in very simple solutions containing inorganic nitrogen, a sugar and mineral salts, it is well known that others are more exacting in their requirements and complex mixtures such as peptone, protein hydrolysates and tissue extracts must be supplied for their successful cultivation. Some of them, indeed, are worthy rivals of the more exacting bacteria in their nutritive requirements. As in the case of the bacteria, a number of suggestions were advanced from time to time that vitamin-like substances were needed by these forms (Linossier 71, Willaman 167, Lepeschkin 70). At the time, fifteen to twenty years ago, the evidence for this was necessarily vague. More recently, with increasing knowledge of the vitamins and particularly of the vitamin B complex, this suggestion has been subjected to more direct experimental proof.

*Nematospora gossypii* was one of the first to be investigated systematically. Farries and Bell (30) reported that it required an "accessory factor" which could be obtained in impure form from egg white, crude casein and other sources. Its exact chemical nature was not determined. This work was confirmed by Buston and Pramanik (16), who separated the factor into two fractions by precipitation with barium hydroxide and alcohol. Neither fraction was active in the absence of the other. The active component of one fraction was identified as *i*-inositol, while the other was concentrated to a considerable degree but not identified (15). In the presence of inositol and a "second accessory factor" from lentils *N. gossypii* grew on a medium whose sole nitrogenous constituent was asparagine or ammonium aspartate (15a). It seems likely that this second fraction contained the biotin of Kögl or a mixture of biotin and thiamin, for Kögl and Fries (58) secured growth of *N. gossypii* in a synthetic medium containing these substances together with *i*-inositol.

Recent studies, particularly those of Schopfer, have emphasized the importance of thiamin for a number of the fungi. Growth

and zygospore formation of *Phycomyces blakesleeanus* (a *Mucor*) took place in a synthetic medium following the addition of crystalline thiamin. In fulfilling the nutrient requirements of this mold, thiamin satisfactorily replaced concentrates prepared from yeast or other sources (129, 12). Riboflavin showed no such effect (12). This work was soon extended to include additional species of molds (130, 131) belonging to the genera *Absidia*, *Parasitella*, *Mucor*, *Pilaria* and others, many of which were found also to require thiamin. In contrast to these results *Rhizopus* was inhibited. Results obtained with natural thiamin were shortly confirmed by the use of a synthetic preparation, and maximum growth of *Phycomyces* was secured upon the addition of 0.5 microgram to 25 cc. of medium (132).

In the investigation of other sources of growth-promoting substances, Schopfer (131) reported that wheat-germ extract apparently contained at least one other factor in addition to thiamin, for small amounts of the extract produced rapid development of *Rhizopus*. He also found (133) that extracts of the leaves of many species of higher plants supplied substances producing a similar effect on *Phycomyces*. This material could be extracted with alcohol, was thermostable in acid solution and was adsorbed by fuller's earth and animal charcoal. In a further study of wheat-germ, Schopfer and Moser (139) described procedures for separation and concentration of several factors showing activity for both *Phycomyces* and *Rhizopus*, especially two factors which they designated "MR" and "MP." Thermostability, resistance to alkali and adsorption by animal charcoal were useful for differentiation. It was suggested that MP might be a disintegration product of thiamin. Certain other preparations or definite compounds could not replace the wheat-germ factors. Thus, pantothenic acid, either alone or with *D*-inositol, was without effect on *Phycomyces* and furthermore it did not augment the action of thiamin. Also heteroauxin (3-indoleacetic acid) had no effect on *Phycomyces* or *Rhizopus*.

Nielsen and Hartelius (103) reported that *Rhizopus* cultures produce a substance, "Wuchsstoff B," stimulating the growth of *Aspergillus*. A stimulant in beer wort for *A. niger* and for yeasts



was later subdivided into several components (104). Certain of these stimulants were required for yeasts and were easily oxidized while others affected mold development and were more resistant to oxidation by hydrogen peroxide and potassium permanganate. The presence of metals (co-growth substances) was also emphasized by Nielsen (102). The influence of growth factors upon development and nitrogen assimilation of *A. niger* was studied by Bünning (11). Thiamin as a rule exerted little growth-promoting effect, while riboflavin led to an increase of about 30 to 40 per cent in the dry weight of the mycelium, though amounts of about 4 to 20 micrograms per cubic centimeter were necessary to bring about this effect. Both of the vitamins as well as several unknown growth factors promoted absorption of nitrates by the mold and this in turn was connected with an intensified respiration.

Mosher, Williams and associates (90) studied the nutritional requirements of *Trichophyton interdigitale*. In addition to rather specialized requirements with respect to inorganic ions and amino acids, this fungus apparently requires at least four accessory substances for satisfactory development. These are: thiamin, riboflavin, *i*-inositol and Williams' pantothenic acid.

A study of the requirements of a number of different fungi, including representatives of the Phycomycetes, Ascomycetes and Basidiomycetes was made by Kögl and Fries (58). Biotin, thiamin and *i*-inositol were tested in a basal medium of glucose, tartrate, and inorganic salts. The thiamin requirement of *Phycomyces* was confirmed. In addition a number of species of the Ascomycetes and Basidiomycetes were found either to require thiamin or to be stimulated by it. A few exceptions to this requirement were also encountered, thus *Nematospora gossypii* required biotin and *i*-inositol for appreciable growth which was further increased by addition of thiamin, while *Lophodermium pinastri* needed biotin and thiamin.  $\beta$ -Alanine was without effect on these types. Kögl and Fries believed that in those cases where a particular factor was found to be unnecessary it was synthesized by the mold. Pairs of fungi with complementary requirements could be grown on a medium without any of the

three factors, although growth was slower under these conditions.

Several recent reports have dealt with the effect on molds of the components of the thiamin molecule. The need for the pyrimidine and thiazole components appears to differ with various molds. In the study of *Phycomyces*, Schopfer and Jung (138) reported that each of these components alone had little or no effect but in combination the effect was identical with that of the whole thiamin molecule. Similar results were reported by Sinclair (143) who found also that thiamin diphosphate (cocarboxylase) was about as active as the vitamin itself. Robbins and Kavanagh (124) found that a mixture of the thiazole component with a 5-bromomethyl derivative of the usual pyrimidine was as effective as molar equivalent amounts of thiamin. The vitamin was therefore believed to be synthesized by the mold from the separate components, since they were required in molecularly equivalent quantities. Additional data on the effectiveness of derivatives of thiamin and its two components with respect to microorganisms in general will be discussed in a later section.

Thus the impure fractions from plant and animal tissues can be replaced in a few instances by small amounts of definite chemical substances. Of the compounds thus far demonstrated to possess growth-promoting activity for the fungi, thiamin assumes an important part, as it does with the bacteria and higher forms of life. Presumably many of the parasitic fungi are unable to synthesize this molecule or one of its components and so fail to develop. Also, there is evidence of the need for a number of interacting factors, the absence of any one of which may lead to complete failure or a marked retardation in development. Inositol seems to be needed in some instances; likewise the preparations known as biotin and pantothenic acid. Future work will doubtless supply further evidence concerning the nature of these substances and bring to light still others now unrecognized.

#### MISCELLANEOUS STUDIES

In addition to the work on groups of bacteria treated in the preceding sections, several other studies may be mentioned here.

From peptone and from blood, Sahyun, Beard and associates (125) obtained in partially purified form "activators" which stimulated cell multiplication of *Escherichia coli*. This effect was in addition to that exerted by known amino acids, and the activating substance was not destroyed by growth of the organisms in media containing it. Dunn and Salle (24) extracted stimulating agents from rice bran with 60 per cent methanol and 25 per cent ethanol. Evidently the rice bran extract also contained food material and inorganic salts. The growth of carbohydrate-fermenting organisms was greatly enhanced and it was suggested that the stimulating agent might be carbohydrate in nature, but was not glucose.

Koser, Chinn and Saunders (60) found that certain gelatins contain growth factors for many of the commoner pathogens, including such types as hemolytic streptococci from scarlet fever, pneumococci, *Brucella* and others. In a synthetic medium, in which these organisms were unable to develop, the addition of some gelatins promoted ready growth of these types. A more highly purified photographic gelatin did not support growth under the same conditions.

*Protozoa.* While no attempt has been made to review exhaustively the literature dealing with the protozoa, several instances may be cited to show the importance of the accessory growth factors for development of certain of these forms. M. Lwoff and A. Lwoff (80, 73) found that hematin, protohemin, and protoporphyrin could replace an essential substance supplied by blood for cultivation of several trypanosomes of the genera *Strigomonas* and *Leptomonas*. Since protoporphyrin contains no iron the trypanosomes can evidently combine this molecule with traces of iron present in the medium and thus construct the iron-containing hematin. Lwoff and Dusi (74) and Lwoff and Lwoff (82, 75) have shown that a number of different forms (*Polytomella caeca*, *Polytoma caudatum*, *P. ocellatum*, *Chilomonas paramecium*, *Glaucoma piriformis* and *Strigomonas oncopelti*) need thiamin. In addition one or more other factors are probably required by some of these types. According to a recent report (83) *Schizotrypanum cruzi* requires ascorbic acid and hematin.

## THE ROLE OF INORGANIC SALTS IN PROVIDING GROWTH-PROMOTING EFFECTS

One explanation of the growth-promoting properties of tissue extracts is based on the assumption that the effect may be due to the presence of certain inorganic salts, which are needed by the microorganism. Of the enormous literature dealing with the effects of inorganic salts upon microorganisms, the following may be cited as bearing more particularly upon our subject. Webster and Baudisch (163) and Baudisch (4) stressed the importance of certain "active" forms of iron salts and iron oxides which might function as the X factor in the growth of hemophilic bacteria. Reed and Rice (118) secured heavier growth of the tubercle bacillus and of several related acid-fast types in a synthetic medium when small amounts of iron and citrate were added. The citrate prevented precipitation of the iron. Elvehjem (26) emphasized the importance of iron and copper in the growth and metabolism of yeast and suggested that a considerable part of the beneficial action of bios depended upon changes which made iron more available for assimilation. Greaves, ZoBell and Greaves (42) reported that growth of yeasts in a mineral salt-sugar solution was increased by minute amounts of iodine. Richards (119) stressed the importance of thallium and expressed the belief that this element may be one of the growth stimulants for yeast that have been referred to as bios. Thallium in varying amounts was present as an impurity in different brands of asparagine.

Burk, Lineweaver and Horner (13) reported that growth stimulation of *Azotobacter* by humic acid was due to the iron content of the latter and that natural humic acid could be replaced by several organic or inorganic iron compounds. Thorne and Walker (156) found that growth of several species of *Rhizobium* in a purified sucrose-nitrate medium was greatly increased by the addition of small amounts of iron, especially ferric chloride. The importance of molybdenum and zinc for development of *Aspergillus niger* was emphasized by Steinberg (147). The decreased yield of mold growth obtained when purified sucrose was used in a synthetic medium was interpreted as being due to the removal of small amounts of molybdenum and zinc from the

sucrose, rather than to the removal of bios or other accessory growth substances.

These references and others of a similar nature present an impressive argument for the inorganic salts, and it is not surprising that a number of the foregoing workers expressed doubts of the existence of accessory growth substances of organic nature in yeast decoctions or tissue extracts.

On the other hand, there is evidence that ashing of tissue preparations destroyed the growth-promoting effect. The writers (66, 126) found that ashing of active fractions obtained from veal infusion, liver, spleen, yeast and white potatoes caused a complete loss of the growth-promoting property. Schopfer and Moser (139) in studying the factors in wheat germ for molds state that the mineral substance present in the ash of several extracts was not responsible for the growth-promoting effect. Tatum, Peterson and Fred (150) ashed the Neuberg filtrate fraction in connection with their work on propionic acid bacteria and found that the ash did not produce the stimulative effect of the original extract. Clark (17) found that ashing and wet combustion destroyed the growth factor for *Rhizobium*. M. Lwoff (81) reported that "active" iron compounds, as employed by Baudisch for *H. influenzae*, were not effective as substitutes for hemin in supplying the needs of the trypanosome *Strigomonas fasciculata*. The writers and their associates (62) were unable to demonstrate any growth-promoting effect when various amounts and combinations of inorganic salts, particularly those of the heavy metals, were substituted for active growth-factor preparations from tissues.

Concerning the importance of the inorganic salts and particularly of the metals which act as catalysts in biological systems there can be no doubt whatsoever. It is unfortunate that our knowledge of the mineral requirements of microorganisms is so incomplete that we are continually uncertain, when attempting cultivation in simplified media, whether the proper compounds or the proper amounts have been supplied. However, this objection has been met by some workers who have employed the ash of biological materials which support growth.

It has been common practice to ignore the traces (or perhaps larger amounts) of these compounds which are present as impurities with the amino acids, sugars and other ingredients used for synthetic media. Glassware, metallic filters and other sources contribute an additional supply.

Doubtless, if we knew more of the mineral requirements of the microorganisms our efforts to obtain satisfactory and rapid growth of the fastidious types in synthetic media would be more successful. Aside from these important inorganic ingredients, however, recent work has revealed the significance of organic entities which are essential for the development of some of the more exacting bacteria, yeasts and molds. It would appear, therefore, that the basic idea of searching for such organic compounds need not be altered, but that along with such endeavor there should be an alert recognition of the importance of the inorganic constituents.

#### GROWTH-PROMOTING EFFECTS AND REMOVAL OF INHIBITING AGENCIES

Another explanation for the growth-promoting effects which follow the addition of tissue extracts to a simplified medium is that the added organic matter has combined with certain "toxic" or inhibitory substances present in the medium, thereby removing a harmful agent which previously restrained cell proliferation. This suggestion was advanced by Fernbach (31) and Windisch (174) in the early discussions on the effect of bios on yeast growth, and it has since appeared from time to time in connection with the studies on bacteria. Windisch in particular called attention to the presence of copper in distilled water and in media.

It seems unnecessary to review here the many reports dealing with possible inhibitory effects of the varied components of culture media. One example, taken from the more recent literature, will serve as illustration. O'Meara and Macsween (106, 107) found some commercial peptones contained sufficient copper to inhibit growth in ordinary nutrient broth when the inoculum consisted of only small numbers of cells. The addition of blood serum to the medium rendered it suitable for growth, presumably

by combining with or precipitating the copper. Here is an excellent example of apparent growth-promoting or growth-stimulating effect following the addition of blood serum. While the possibility of such effects must always be kept in mind, there now appears to be ample evidence that growth factor activity cannot be accounted for solely on this basis.

#### GROWTH-PROMOTING EFFECTS RESULTING FROM CHANGES IN PHYSICAL PROPERTIES OF THE MEDIUM

In the attempts to develop suitable culture media for the more exacting microorganisms there is evidence that the physical character of a medium is not only important, but at times may be *the* factor determining suitability of the medium. With respect to the study of growth-promoting factors, various investigators have attributed the beneficial effect of tissue extracts to changes produced in the physical character of the medium.

Differences in hydrogen-ion concentration, surface tension, osmotic pressure, and the oxidation-reduction potential are among the more obvious alterations which may result from the addition of tissue extracts or other growth-factor preparations. Of these various properties the importance of a suitable pH is well recognized, and the oxidation-reduction potentials of culture media and of developing cultures have received serious study. Less attention has been paid to the other properties. Since several publications have emphasized particularly the possible misinterpretations of growth-factor effects due to changes in the oxidation-reduction potentials of the culture medium, most of our discussion here will be concerned with this aspect of the problem, but it must be realized that the same principles apply to the other physical properties.

There is now considerable evidence that bacteria can multiply only in media where the redox potential is within certain limits and that the limiting zone, whether broad or narrow, varies with the individual organisms. The favorable conditions for growth which are brought about by various procedures, such as the addition of tissue extracts, large inocula, boiling of the medium, etc., have been attributed, at least in part, to the reduction of oxidized

substances or to the establishment of a suitable reduction potential in the medium.

Wright (177, 178) called attention to inhibitory properties of the usual peptone-infusion media, particularly when seeded with small numbers of cells, and attributed this effect to constituents of peptone in the oxidized state. Heating the peptone solution with meat, during the course of preparation of the medium, improved its growth-promoting properties, and Wright believed this effect was due to reduction of the peptone, or certain of its constituents, thereby removing the toxic action. He also suggested that the inhibitory effect must be taken into account in experiments relating to accessory growth factors. Dubos (23) reported the presence in peptone of substances which were bacteriostatic in the oxidized state. Their bacteriostatic action could be overcome by the addition of thioglycollic acid.

Allyn and Baldwin (3) have also emphasized the importance of the oxidation-reduction character of media in the initiation of growth. A yeast-mannitol medium supported growth of *Rhizobium* when inoculated with small numbers of cells, while in a nitrate-mannitol medium no growth occurred unless very large inocula were used. The yeast medium was more reducing in nature than the nitrate-mannitol medium. The nitrate-mannitol medium permitted growth with similar small inocula after the addition of thioglycollic acid, powdered agar, or other reducing agents. In this instance a synthetic medium, upon the addition of reducing agents, supported growth as readily as a yeast medium. Thorne and Walker (156) found that the addition of reducing agents such as cysteine or thioglycollic acid increased growth and oxygen utilization of *Rhizobium* in media composed of highly purified ingredients (nitrate, sucrose, and inorganic salts). Cysteine brought about increases comparable to those induced by brown sugar, which has been said to contain appreciable quantities of accessory factors. They found no evidence that root nodule bacteria require any complex, unidentified substances for their growth. From these reports it is evident that a growth-promoting effect may be the result of adjustment of the oxidation-reduction potential from a less to a more favorable



region, or from the reduction of oxidized ("toxic") substances in the medium.

The importance of CO<sub>2</sub> tension in the cultivation of bacteria has been stressed by many workers and has been well reviewed by Knight (52). The effect of other changes in the physical character of the medium has also been reported. Hitchens (44) recommended the addition of 0.1 per cent agar to ordinary broth. In the resulting semi-solid medium a number of the more fastidious types developed more luxuriantly than in broth or on ordinary solid agar slants. Another interesting example has appeared in studies on methane fermentation. Breden and Buswell (8) found that addition of shredded asbestos to a liquid medium provided a suitable background for development of the methane-producing types which appeared to require the presence of finely-divided material in suspension. With the shredded asbestos in place of sewage sludge, subcultures could be carried through many transplants.

While it is true that many of the studies on growth-promoting substances have ignored possible changes in oxidation-reduction potential and other physical characteristics of the medium, the growth-promoting effects observed probably are not due to physical changes. In the study of *Lactobacillus delbrückii*, Snell, Tatum and Peterson (145) noted that the addition of potato extract lowered the oxidation-reduction potential of the basal medium and produced a growth-stimulating effect. However, substitution for the potato extract of agents such as cysteine, cystine or thioglycollic acid, which lowered the potential in like amount, did not produce the stimulating effect. Rahn and Hegarty (114) found that substances used to lower the redox potential failed to stimulate and at times even slightly retarded acid production by *Streptococcus lactis*. Koser, Saunders and associates (62) found that changes in the physical properties of the test medium suggested by the foregoing reports did not produce the growth-promoting effect shown by extracts prepared from tissues.

In studying *Streptothrix corallinus* in a synthetic medium plus tissue concentrates, Reader (115) found that alterations of the

surface tension of the fluid, within ordinary limits, did not affect the amount of growth and concluded that the growth-promoting activity of the added concentrates was not due to lowering of tension of the medium.

Our evaluation of these conflicting viewpoints leads to a conclusion similar to that expressed in the previous discussion on the effects of inorganic salts. There can be no doubt of the importance of the physical character of the medium. Unfavorable levels of redox potential or other less well-recognized properties may prevent cell proliferation as effectively as unfavorable ranges of hydrogen-ion concentration. In some cases an apparent growth-promoting effect may well have been due to the alteration of such conditions. Unfortunately, we are still quite vague as to what many of the physical specifications should be and so the whole subject is left in a rather uncertain state. It seems doubtful, however, that the growth-promoting effects of minute amounts of such compounds as thiamin, nicotinic acid, and  $\beta$ -alanine can be explained as due to a change in the physical properties of the medium.

#### DEFINITE COMPOUNDS WHICH SHOW GROWTH-PROMOTING ACTIVITY

By way of summary, the compounds which have been substituted successfully for the complex mixtures of plant and animal tissue extracts are listed in table 1. Only those substances which seem to fill a fundamental and often specific need for cell proliferation are included. With one exception, the chemical structure of all of these compounds is now definitely known.

*Hematin or hemin.* This iron-containing compound needs little comment here since it has been discussed in earlier reports. It appears to have been the first of the so-called accessory substances for microorganisms to be definitely identified. In addition to its important rôle in the cultivation of bacteria, it has also been shown to substitute for a component of blood in the cultivation of several trypanosomes.

*i-Inositol.* The inclusion of *i*-inositol in our list may be open to some question since by itself it is not sufficient for cell multipli-

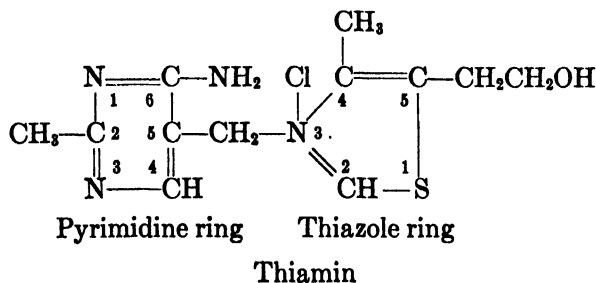
cation, but the presence of one or more "cofactors" is necessary. Furthermore it usually must be supplied in larger amounts than

TABLE 1  
Compounds which show growth-promoting activity

SUBSTANCE	ORGANISM	COFACTOR	REFERENCE
Hemin.....	<i>Hemophilus influenzae</i>	"V" factor	(20, 21, 153, 154, 155, 32)
i-Inositol.....	<i>Saccharomyces</i>	Other unknown substances	(25)
i-Inositol.....	<i>Nematospora gossypii</i>	Other unknown substances	(16, 58)
Thiamin.....	Molds	Other unknown substances	(129, 12, 130, 131, 58)
Thiamin.....	Propionic acid bacteria	Ether sol. factor from yeast	(152)
Thiamin.....	Yeasts	i-Inositol	(172)
		"Pantothenic acid"	(170, 171)
		"Biotin"	(59)
Thiamin.....	<i>Staph. aureus</i>	Nicotinic acid	(53)
		Nicotinamid	
Riboflavin.....	Lactic acid bacteria	Hydrolyzed casein	(175, 108)
		Ether sol. factor from yeast	
Nicotinic acid and derivatives.....	<i>Staph. aureus</i>	Thiamin	(53, 68)
Nicotinic acid and derivatives.....	<i>C. diphtheriae</i>	$\beta$ -Alanine	(95)
Nicotinic acid and derivatives.....	<i>Shigella paradysenteriae</i>	None	(61)
Cozymase.....	<i>Hemophilus parainfluenzae</i>		(76)
$\beta$ -Alanine.....	<i>Saccharomyces</i>	Aspartic acid	(171, 87)
		Inositol	
		"Pantothenic acid"	
		Thiamin	
		Leucine	
$\beta$ -Alanine.....	<i>C. diphtheriae</i>	Nicotinic acid	(97, 63)

the other growth factors. It has been included, nevertheless, because it represents one of the few instances where a definite compound has been identified as the active ingredient of a growth-

promoting preparation. Its function in cell metabolism seems uncertain at the present time. According to Eastcott (25) it is stored in the cells, since the inositol taken up by yeast from the culture medium can be quantitatively recovered by hydrolyzing the yeast crop.



*Thiamin.* Studies of the two ring structures which compose the thiamin molecule have revealed an interesting diversity of requirements among those microorganisms for which this substance is effective as a growth factor. In a few instances the *intact* thiamin molecule is required. The pyrimidine and thiazole components when supplied as separate entities, in equivalent molar concentrations, are ineffective as a substitute for the whole molecule. The two components cannot substitute for thiamin in the case of the protozoa *Strigomonas oncopelti* and *Glaucoma piri-formis* (82, 75). A similar need for the intact thiamin molecule has been reported for certain of the parasitic fungi, namely several species of *Phytophthora* (123a) and the basidiomycete *Ustilago scabiosae* (136). For the related *U. violacea* thiamin can be replaced partially by the two components. Evidently these microorganisms are unable to put together the two components to form the whole thiamin molecule or, in the case of *U. violacea*, this synthesis is accomplished too slowly to permit normal development.

Other types are somewhat less exacting in their requirements. *Phycomyces blakesleeanus* requires both components of the thiamin molecule but not the intact molecule itself (138, 143, 124). This is also true of *P. nitens* (124a), *Staphylococcus aureus* (54) and the flagellate protozoan *Polytomella caeca* (74). It appears that these

microorganisms are not able to synthesize either of the two component ring structures. The molds *Absidia ramosa* and *Parasitella simplex* require both components for rapid development but can grow more slowly in the presence of the pyrimidine constituent alone (134). Apparently the thiazole is synthesized by these molds, but in an amount insufficient for normal growth.

Still other microorganisms can develop as readily in the presence of only one of the components as when the whole thiamin molecule is supplied. This is true of *Mucor ramannianus* (100) which needs only the thiazole constituent and also of the yeast, *Rhodotorula rubra* (135) and several higher fungi which require only the pyrimidine constituent (124a). There is some evidence that the component which is not required is synthesized by the organisms.

In contrast to the foregoing are the many microorganisms which are able to develop in a synthetic medium devoid of thiamin. While our knowledge of the physiology of these types is still quite incomplete, it appears probable that thiamin plays an important rôle in their metabolic processes. Since in these cases neither thiamin nor its direct components are supplied, these organisms apparently possess the property of synthesizing the two ring structures from much simpler compounds.

*Derivatives of thiamin and its components.* There is evidence of a high degree of specificity in the chemical structure of the active compounds. Thiochrome, an oxidation product of thiamin in which the nitrogen atom of the 6-amino group of the pyrimidine is linked to the 2-carbon atom of the thiazole ring, can substitute for thiamin only very imperfectly or not at all for growth of *Staphylococcus aureus* (54), *Phycomyces* (137) and *Rhodotorula rubra* (134). Also, a molecule similar to thiamin but lacking the  $\beta$ -hydroxyethyl group at the 5-position of the thiazole ring was inactive for *Staphylococcus* (54). Substitutions in various positions of the pyrimidine ring of the intact thiamin molecule greatly reduced or abolished the activity of thiamin (55a). The activity could be restored by addition of the normally substituted pyrimidine.

Several substitution products of both the pyrimidine and the

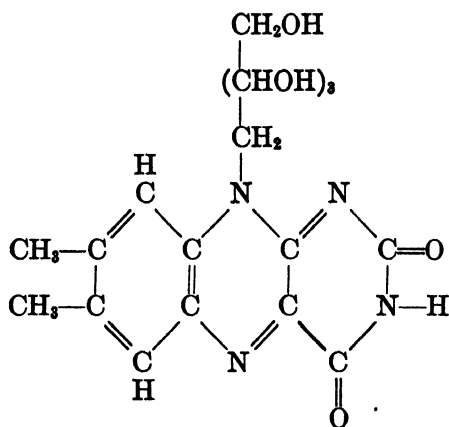
thiazole components have also been tested. 2-Methyl-5-amino-methyl-6-aminopyrimidine<sup>2</sup> was active for *Staph. aureus* in the presence of the thiazole component, while under the same conditions 2-methyl-5-hydroxymethyl-6-hydroxypyrimidine, 2-methyl-5-aminomethyl-6-hydroxypyrimidine and 2-hydroxy-4-aminopyrimidine (cystosine) were all inactive (54). When the 5-aminomethyl group in 2-methyl-5-aminomethyl-6-aminopyrimidine was replaced by a 5-thioformamidomethyl group, the compound retained activity, though in somewhat lessened degree, for *Staphylococcus* (54) and for *Phycomyces* (143) and was said to "substitute fully" for growth of *Rhodotorula rubra* (134). Upon substitution of a 5-bromomethyl for the 5-aminomethyl group, growth-promoting activity was retained for *Phycomyces* (in the presence of the thiazole component) (124). In a later report Knight and McIlwain (55a) used additional substituted pyrimidines and found that most of them were inactive for *Staph. aureus*. The groups attached to the ring which appear essential for activity are: a methyl group at position 2, an amino group at position 6 and a methyl group substituted in certain ways at position 5. Thus at position 5,  $-\text{CH}_2\text{NH}_2$ ,  $-\text{CH}_2\text{OH}$  and  $-\text{CH}_2\text{NH}\cdot\text{CSH}$  permitted growth, but  $-\text{CH}_3$  and  $-\text{CH}_2\text{CO}\cdot\text{NH}_2$  were inactive. Nucleic acid or hydrolysates of nucleic acid which supply pyrimidines were not effective for *Phycomyces* when substituted for the specific pyrimidine (124).

Substitutions in the thiazole component have demonstrated a similar high degree of specificity. For growth of *Mucor ramanianus* 4-methyl thiazole, 4,5-dimethyl thiazole and 2-mercapto-4-methyl thiazole were all unable to take the place of the usual 4-methyl-5-hydroxyethyl thiazole (100). For growth of *Phycomyces*, Robbins and Kavanagh (124) found that a number of other thiazole derivatives were ineffective as substitutes for the usual component. Likewise a number of sulphur-containing compounds such as methionine, glutathione, thioglycollic acid and others were ineffective. For growth of *Staph. aureus* Knight

<sup>2</sup> The designation of the pyrimidine derivatives has been changed in this article to conform with the usual system of numbering the positions in the pyrimidine ring.

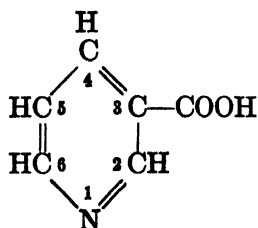
and McIlwain (55a) found other substituted thiazoles were either inactive or showed reduced activity.

Judging from the relative effects on *Staph. aureus* of the different substituted groups at position 5 of the pyrimidine, it appears probable that the pyrimidine and thiazole components are joined to form the intact thiamin molecule, rather than that the two components are used separately (55a). Certain observations of Hills (43a) on pyruvate metabolism by *Staph. aureus* support this hypothesis, as does the work of Robbins and Kavanagh with *Phycomyces* (124).

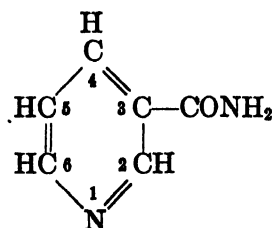


Riboflavin

*Riboflavin.* Little information concerning riboflavin appears to be available aside from that given in the references previously listed. Other related compounds possessing the isoalloxazine or alloxazine rings have not been available or their activity has not been tested in bacteriological work.



Nicotinic Acid



Nicotinamide

*Nicotinic acid and nicotinamide; coenzyme; cozymase.* The recent work of Warburg and of Euler and their associates demonstrated that nicotinamide is a constituent of the coenzyme<sup>3</sup> from horse blood and the chemically related cozymase<sup>3</sup> from yeast. The nitrogen in the pyridine ring of nicotinamide is important in the transfer of hydrogen in biological oxidations. It is of interest that in the case of *H. parainfluenzae* (76), the pyridine nucleotide di- or tri-phosphate was required and nicotinic acid, nicotinamide, and adenylic acid (adenine + d-ribose + phosphoric acid) could not substitute as growth factors. In the case of the staphylococcus, the diphtheria bacillus and the dysentery bacilli only the nicotinic acid or its amide was needed.

The comparative activity of nicotinic acid, nicotinamide and some related compounds has been studied in a few cases. According to Mueller (95) the amide was about one-tenth as effective as the acid for the diphtheria bacillus. In studies on the staphylococcus Knight (53) found the amide to be about five times more potent than nicotinic acid in the presence of appropriate amounts of thiamin. Methyl nicotinate also was effective but development of cultures was slower. Pyridine-3-nitrile was ineffective as such but was active after hydrolysis (yielding nicotinic acid). In a later publication Knight and McIlwain (55a) reported that the following compounds were all inactive: coramine, pyridine-3-sulfonic acid,  $\beta$ -picoline, nicotine, trigonelline methyl sulphate, trigonelline chloride, isonicotinic acid, picolinic acid, quinolinic acid, 2,4-dimethylpyridine-3,5-dicarboxylic acid and 2,4,6-trimethylpyridine-3,5-dicarboxylic acid. Landy (68) has reported that the two isomers of nicotinic acid (picolinic and isonicotinic acids) cannot replace nicotinic acid for the growth of *Staph. aureus*;  $\alpha$ - and  $\gamma$ -picoline were also ineffective. Nicotinamide and the N-ethyl nicotinamide were both active but the N-diethyl compound was inactive. Sodium and ammonium nicotinate were as effective as nicotinic acid itself, but the ethyl ester of the acid was slightly less active.

In studies on the dysentery bacilli Koser, Dorfman and Saun-

<sup>3</sup> Constituents of coenzyme and cozymase are adenine, nicotinamide, 2 molecules pentose and either 3 or 2 molecules, respectively, of phosphoric acid.



ders (61) found the amide to be slightly more effective, but the difference between the amide and the acid was not marked. In a more extended study of nicotinic acid derivatives Dorfman, Koser and Saunders (22a) showed that pyridine-3-sulfonic acid, trigonelline, 6-methyl-nicotinic acid, nipecotic acid, isonicotinic acid,  $\beta$ -acetylpyridine,  $\beta$ -picoline, and pyridine were devoid of growth-promoting activity. The following substances favored good growth in the dilutions indicated: nicotinic acid, nicotinamide, methyl nicotinate  $M \times 10^{-7}$ ; trigonelline amide, ethyl nicotinate, nicotinuric acid, ethyl nicotino-acetate  $M \times 10^{-6}$ ; nicotinic acid N-methyl amide  $M \times 10^{-5}$ ; nicotinonitrile  $M \times 10^{-4}$ . Picolinic acid and quinolinic acid showed activity at a dilution of  $M \times 10^{-4}$  but there is a possibility that these two preparations may have been contaminated with traces of nicotinic acid.

*Beta-alanine.* The growth-promoting activity of this amino acid for some yeasts and the diphtheria bacillus is quite in contrast to the negative results secured with the ordinary  $\alpha$ -alanine. Many of the protein hydrolysates or basal synthetic media in which the diphtheria bacillus has failed to develop have contained  $\alpha$ -alanine. Addition of one microgram or less of  $\beta$ -alanine per cubic centimeter of medium fills some need for cell multiplication which is not supplied by the  $\alpha$ -form. This importance of a  $\beta$ -amino acid is of particular interest since in the past biologists and chemists have considered the  $\alpha$ -amino acids as being the only ones of biological importance. In view of the incomplete knowledge of the composition of proteins and other tissue extractives, it may well be that  $\beta$ -amino acids play a far more important rôle than has heretofore been recognized.

The diphtheria bacillus is capable of obtaining  $\beta$ -alanine from naturally occurring *l*-carnosine but not from the *d*-form (Mueller, 96). Upon acid hydrolysis, both *d*- and *l*-carnosine yield equally active products.

It might also be added that asparagine and aspartic acid which have been commonly used in synthetic media, can yield  $\beta$ -alanine and it is quite possible that many organisms capable of developing in the simpler synthetic media can bring about this change and

secure  $\beta$ -alanine from asparagine. In other words, the need for  $\beta$ -alanine may be much more wide-spread among microorganisms in general than indicated by the results with some yeasts and the diphtheria bacillus, but many types may secure it from asparagine or other sources.

*Biotin.* Tentative empirical formula,  $C_{11}H_{18}O_3N_2S$ . This substance has been included in the list of growth factors although its structural formula is not yet known. From the reports of Kögl and associates (57) it appears to be a definite entity. It is an amphoteric substance and its methyl ester has been obtained in crystalline form. The evidence thus far submitted seems to show that it is important in the cultivation of a number of microorganisms belonging to quite different groups and that very minute amounts of it exert a distinct growth-promoting effect.

#### SYNTHESIS OF ACCESSORY GROWTH FACTORS. MUTUAL INFLUENCES

It seems reasonable to believe that many of the growth factors listed in the foregoing section and others still unrecognized are required by microorganisms in general. For many types there is no need to supply them as such, because the organisms presumably can synthesize them from simpler substances. Here and there, however, we encounter a type which is unable to synthesize *one* of a number of required substances (e.g., dysentery bacilli and nicotinic acid). When this one compound is supplied along with needed sources of nitrogen, energy and inorganic salts, rapid multiplication ensues. Other organisms happen to be unable to manufacture *two* of these substances (e.g., the staphylococcus with respect to thiamin and nicotinic acid) and do not develop unless both are supplied. Either compound in suboptimum amount limits growth. Again, an organism may be totally unable to synthesize one needed compound but can construct another required substance slowly—too slowly for normal growth. Here one substance is essential and another serves to stimulate growth. In a similar way, other more exacting microorganisms will doubtless be found to need an assortment of various substances which they themselves cannot produce. Thus,

there are a number of interacting compounds and often the action of any one becomes evident only in the presence of the others. It is obvious, too, that the building material which is available for the organism will doubtless vary from one situation to another so that the kind of "raw product" offered may often determine in large measure whether or not certain compounds can be synthesized.

It is believed that the lack of synthetic abilities, with resulting "fastidiousness" of the organism, represents a loss of properties in connection with adaptation to a commensal or a parasitic mode of life and that it is not due to the acquisition of new growth requirements (52, 76). In nature those organisms which are unable to accomplish such syntheses must depend upon production of the required compounds by other types. Many of the instances of growth stimulation of one type by another, seen on the ordinary laboratory media, can doubtless be explained on this basis.

The familiar "satellite" phenomenon of Grassberger (41), who called attention to the increased size of colonies of *H. influenzae* when growing in close proximity to colonies of staphylococci, has been often encountered with many other species. A few instances associated with definite growth factors follow. In the work on thiamin Müller and Schopfer (100) found a mold (*Mucor ramannianus*) which was incapable of synthesizing one component of the thiamin molecule and so was unable to develop unless this structure was supplied. A yeast (*Rhodotorula rubra*) needed only the other thiamin component. These organisms were capable of developing together in a simple medium, without any added thiamin, since each manufactured the particular component of the thiamin molecule needed by the other. Another instance was reported by Kögl and Fries (58) with respect to *Polyporus adustus* and *Nematospora gossypii*. Neither of these fungi was able to grow in a synthetic medium in pure culture; when inoculated together, however, they developed. *Polyporus* requires thiamin which was supplied apparently by *Nematospora*, while the biotin requirement of *Nematospora* was supplied by *Polyporus*. With the varied synthetic abilities of diverse organ-

isms and the many situations encountered in nature there would seem to be almost no limit to the number of such combinations.

Other striking relationships have been reported between microorganisms and the higher plants. Of particular interest are those concerning the fungi and orchids, and the relationships between the root-nodule bacteria and legume plants. A review of this aspect of the problem has been given by Bonner (5). A fuller recognition of the limitations of synthetic abilities of organisms would doubtless help in no small degree in explaining some of the baffling symbiotic and other relationships so frequently encountered in nature. The apparent inability of many of the pathogenic microorganisms to synthesize accessory growth factors, such as nicotinic acid and thiamin, seems highly significant in connection with their invasion of the tissues of the higher animals and plants where these substances may be found.

#### FUNCTION OF THE ACCESSORY GROWTH FACTORS

What essential rôle is played in the physiological processes of microorganisms by the minute amounts of these growth substances? A consideration of the substances now known to possess growth-promoting properties shows that most of them enter into the structure of enzymes or coenzymes concerned with cell oxidations. Thus, the pyrophosphoric ester of thiamin, thiamin diphosphate, functions as a cocarboxylase with a protein of yeast cells and in this enzyme system strongly promotes the decarboxylation of pyruvic acid, an important intermediate product in the dissimilation of glucose. Nicotinamide is one of the components of the coenzyme of Warburg and of cozymase which plays an important rôle as a mediator in biological oxidation. Riboflavin when combined with phosphoric acid and protein becomes the "yellow enzyme" of Warburg and Christian which, together with a second enzyme and the coenzyme, brings about the oxidation of hexose-monophosphoric acid ester, an important step in sugar oxidation.

In these cases, the fulfillment of the "growth factor" requirements of one of the more fastidious microorganisms furnishes a portion of an enzyme or coenzyme molecule which the organism

itself cannot synthesize, but which it needs in order to carry on its metabolic processes. Without the needed component the sequence of events in the respiratory chain is broken and cell multiplication is not possible. Since these substances enter into a catalytic respiration system it becomes apparent why such minute amounts suffice.

A similar function has been suggested for hemin (80, 73, 78) in relation to certain trypanosomes and *H. influenzae*. In the past, much attention has been centered on the peroxidase or catalase activity of X factor as a protective mechanism against toxic peroxides. From the work of the Lwoffs, however, it appears reasonable that the need of the hemophilic microorganisms for hemin or X factor is connected with inability to synthesize the prosthetic group of a respiratory enzyme.

In past years, attempts to cultivate the more exacting types in chemically definite media have considered for the most part only the question of structural material for the cell proteins and neglected the materials required for the building of enzyme systems or other special needs. Rahn (113) has suggested that the vitamin-like substances might be needed for construction of certain special molecules in the cell, for example the genes. Since these substances would enter into the structure of only a few molecules in the cell, therefore only very small quantities would presumably be required. It is now apparent that the enzyme-coenzyme systems may be included among the cell constituents for which special structural material is needed.

Of the definite compounds now associated with growth-factor activity for microorganisms, *i*-inositol and  $\beta$ -alanine have not been shown to be components of an enzyme system, insofar as the writers are aware. The interpretation of their rôle in cellular metabolism must await further evidence. In the meantime, it is an interesting thought that the demonstration of the important part which these substances play in development of certain microorganisms may give a clue to their occurrence in some enzyme-coenzyme systems whose composition is now unknown.

The present knowledge of the growth factors, while fragmentary, permits a clearer idea of future lines of work which should

prove to be fruitful, and we are now better able to direct our efforts in solving the mysteries which still surround the growth requirements of many of the microorganisms. If one component of a coenzyme, such as nicotinic acid for example, is needed by a microorganism, perhaps two, three, four or more components of this or other systems may be required by still more exacting pathogens, or by some of the more fastidious types important to agriculture or to the fermentation industries. Following this line of reasoning, it might be assumed that some of the strictest parasites, which multiply only in the presence of living tissue or within living tissue cells, have lost a large measure of constructive ability in connection with their adaptation to such an abode. Such organisms might conceivably be unable to put together a needed organic catalyst even when supplied with its several component parts and perhaps will be found to need the intact, preformed constituents of a whole system.

#### SUMMARY

A number of attempts have been made to isolate the growth-promoting substances known to be widely distributed in animal and plant tissues. In many instances identification of the growth substances has not yet been accomplished, though some progress has been made in their separation. In other cases, however, several compounds of known chemical structure are now recognized as the active substances of tissue extracts. These are: hemin, *i*-inositol, thiamin, nicotinic acid and its amide,  $\beta$ -alanine, riboflavin and pyridine nucleotide phosphate (coenzyme or cozymase). With the exception of *i*-inositol, these compounds are needed only in very small amounts.

The microorganisms for which one or more of these compounds must be supplied are: *H. influenzae* and related types, propionic and lactic acid bacteria, staphylococci, diphtheria bacillus, dysentery bacilli, certain of the true fungi including some of the yeasts, and certain protozoa. On substitution of the required compounds for tissue extracts, it is now possible to cultivate a number of these types in synthetic media.

Another substance, biotin, has been obtained in crystalline

form as the methyl ester. Others have been obtained in a relatively pure state: the "sporogenes vitamin," pantothenic acid, the "L" fraction for lactic acid bacteria.

Microorganisms requiring the foregoing compounds are unable apparently to synthesize them. There is increasing evidence that other less fastidious types are able to construct them from simpler substances. The various constructive abilities of different organisms are significant with respect to symbiotic and other mutual relationships.

With the exceptions of *i*-inositol and  $\beta$ -alanine, the accessory factors are known to enter into the structure of enzyme-coenzyme systems catalyzing oxidation processes.

The growth-promoting effect of tissue extracts cannot be explained solely on the basis of the inorganic salt content or an alteration in the physical properties of the culture medium.

It is significant that recent work has tended to show the close relationship between the nutrition and metabolism of microorganisms and the higher forms of plant and animal life.

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# THE FIBRINOLYTIC ACTIVITY OF HEMOLYTIC STREPTOCOCCI

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The fibrinolytic activity of hemolytic streptococci is a term used to designate the capacity of broth cultures of *Streptococcus hemolyticus* of the beta type to transform the solid clot of normal human blood into a liquid state. The rapid dissolution of human fibrin by hemolytic streptococci is dependent upon the presence in cultures of an extracellular enzymic substance which is excreted by the living organisms. Reports in the literature evidence the fact that the phenomenon has special characteristics of bacteriological and immunological interest.

The fact that the reaction involves a special kind of bacterial product acting upon a special kind of tissue substrate illustrates the particular qualities of streptococcal fibrinolysis. The process

seems to differ from the catabolic action of proteolytic enzymes which reduce complex protein material to split products of relatively simple chemical composition. Furthermore, a possible correlation with bacterial toxins, which are excreted extracellularly, remains uncertain in the present state of knowledge. However, the phenomenon appears to belong to the types of reaction which include enzymes and toxins, and, for this reason, warrants consideration from a biological standpoint and also as a possible agency in the mechanism of infections due to hemolytic streptococci.

It is the purpose of this article to review and to attempt to evaluate, when possible, the published reports concerning fibrinolytic action of streptococci and other bacteria. The editors of *Bacteriological Reviews* have urged their authors "to distinguish between the essential detail and the isolated, vanishing particular." The fulfillment of these conditions is rendered particularly difficult in the present report because of the fact that the investigations have developed only in recent years. From the published articles of numerous investigators, it is possible to define more clearly some phases of the reaction. However, lines of study suggested by certain factors of streptococcal fibrinolysis have yielded results which have served to broaden the scope of inquiry. Since these findings in many instances constitute new data, it is apparent that final conclusions cannot be drawn for the present. All the reports, which this author has encountered, have been included for the purpose of bringing the subject matter up to date, even though the diversity of some individual researches and the fragmentary reports of others renders difficult a critical assay of some of the results.

The terminology used in reference to some phases of the reaction is to some extent unsatisfactory. Certain phrases are perhaps awkward or confusing. However, at the present time, it would appear to be premature to offer a glossary of fixed expressions. In the current state of knowledge it seems desirable to refer to the phenomenon in terms which emphasize certain special conditions, not for the purpose of imposing exact and restricting definitions, but rather to identify the reaction by specifying the particularly striking features.

## I. MATERIALS AND METHODS OF EXPERIMENTAL PROCEDURES

The fibrinolytic reaction of cultures of hemolytic streptococci is readily demonstrable without any unusual precision of technique. The occurrence of the phenomenon has been commonly encountered in tests with large numbers of strains of hemolytic streptococci. However, laboratory conditions which may affect the results, have been noted by several observers. Some of the conditioning factors assume importance only in certain types of technical procedure which will be referred to under appropriate headings. Other experimental details, however, require more careful general consideration because they need to be taken into account in interpreting results. They also indicate some of the biological factors which influence the production of fibrinolysin by the bacterial cells. From the standpoint of critical analysis it is, therefore, advantageous to review, first, data concerning experimental procedures.

One of the interesting aspects of the reaction concerns the source of the materials used in obtaining the lysis of fibrin by cultures. For example, the characteristically rapid and complete liquefaction of fibrin is usually most strikingly demonstrable when, on the one hand, the bacterial constituent of the test consists of cultures of hemolytic streptococci derived from *man*, and on the other hand, the fibrin, which serves as substrate, is also obtained from *man*. Furthermore, negative or inconclusive results are most frequently obtained when cultures isolated from animal sources are tested against human fibrin, or when cultures derived from human infections are tested against animal fibrin. As will be shown later, the findings just mentioned are not absolute, but are dependent upon quantitative as well as qualitative factors.

The experimental conditions under which the clot-dissolving property of cultures is most satisfactorily obtained consist in mixing the cultures with plasma or fibrinogen before inducing clot formation. By this procedure the organisms and their products are disseminated within the body of the clot as it forms, thus affording maximum surface contact between the active bacterial agent and the fibrin substrate.

It has been noted by several observers (Tillett and Garner (65), Hadfield, Magee, and Perry (19), Madison (31), Dack, Woolpert and Hoyne, (4), Schmidt (57), and others) that the time required for dissolution of normal human fibrin by active cultures varies from a few minutes with some strains to a partial effect exerted by other strains during twenty-four hours' incubation. 0.5 cc. of broth culture plus 0.2 cc. of a 1 to 5 dilution of plasma have been frequently used in tests and have given satisfactory results. Some investigators have, for convenience, employed approximate fractions or multiples of the ratio given above. The evidence is clear that the differences in speed and completeness of fibrin dissolution exerted by strains is dependent upon quantitative differences in the amount of fibrinolysin excreted by the cultures. Consequently, it is obvious that the demonstration of the occurrence of lytic action by strains, as well as the degree of potency, may be conditioned by the quantities of reagents selected for use.

The importance of the quantitative factor is also indicated by the report of Madison (33) concerning results obtained following the concentrations of fibrinolysin contained in cultures of several strains. (The methods of concentration will be described later.) Madison found that among 123 strains only 17 per cent were considered to be actively fibrinolytic when the tests were made with 0.5 cc. of broth culture. However, the percentage of demonstrably positive strains was raised to 35 per cent when cultural material which had been concentrated approximately twentyfold was employed.

The quantitative production of fibrinolytic substances by strains is also related to the cultural conditions under which the tests are made. Madison and Taranik (39) compared the curve of bacterial growth with the production of fibrinolysin, and found that "test tube proliferation" of the bacterial cells paralleled the rate of production of the lytic enzyme. Using quantitative titration, they were able to demonstrate lytic activity with cultures after a few hours' incubation and they also noted that the production of fibrinolysin was maximum when the phase of growth was nearest the peak, which was reached after approximately 12 to 14 hours. After this point in multiplication had

been reached, the production of fibrinolysin was markedly retarded, although some of the cultures retained maximum activity after twenty-four hours' incubation. The experiments did not indicate with certainty whether or not enzyme production requires cell division.

Without making quantitative titrations, Tillett and Garner (65) noted gradual deterioration of lytic activity in cultures which were kept in the incubator for several days. Decrease in potency was delayed, but not entirely arrested, by ice-box temperatures. Schmidt (57) found cultures five to six days old to be active, but he did not report measurements of activity.

It may be seen from the findings just cited that fibrinolysis by active cultures may be demonstrated within wide ranges with respect to age of cultures. However, it is also brought out that greatest activity is determined both by the abundance of growth of the strains and by the time in the phase of growth at which tests are made. With bacterial strains of high fibrinolytic activity the qualitative demonstration of lysis requires no special attention to cultural details. However, with strains which elaborate relatively small amounts of fibrinolysin, experience has shown that the extent of multiplication of streptococci—which may be limited in unfavorable media—and the age of the culture may be important factors in determining the results of fibrinolytic tests with individual strains.

There is, also, suggestive evidence that additional elements in culture media, in the nature of accessory substances, may promote or retard the yield of fibrinolysin by streptococci. There are no published reports dealing with this point. However, the author has noted that when selected strains were cultivated simultaneously in samples of culture media containing different ingredients, the lytic potency of individual strains varied, even though the amounts of growth seemed to be comparable in the different kinds of media. When one considers by analogy the effect of culture media on the production by other bacteria of products such as toxins, it seems likely that the specific stimulation or impairment of the elaboration of fibrinolysin may be subject to conditions of the same order.

Whether variations in the potency of cultures depends upon

differences in the number of individual cells of the culture population, which excrete fibrinolysin, or is referable to the amount of the enzyme produced equally, in any single culture, by all the cells, has not been studied. The problem is common to the broader question concerning bacterial adaptation and selection, which, with respect to enzymes, has been discussed by Yudkin (84), who has considered the types of substances responsible for the increase in enzyme content of microorganisms.

According to the classification of bacterial enzymes employed by Karström (25), the fibrinolysin seems to belong to the group designated as "constitutive" enzymes, which do not require the presence of substrate for the production of the enzyme, as opposed to "adaptive" enzymes which are formed only in the presence of the specific substrate. No study has been made of the effect of the introduction of fibrin into cultures on the yield of fibrinolysin by the bacterial cells. *In vivo*, the possibility that the fibrin of inflammatory exudate might promote the production of fibrinolysin by the infecting organism is suggested by the occurrence of highly potent fibrinolytic strains in widespread infections.

Even though information concerning the effect of environment in the production of fibrinolysin is limited, it has been the common observation of many investigators that whereas many strains during artificial cultivation retain, as a constant property, the fibrinolytic potency exhibited in the initial tests, other strains have not maintained a uniform degree of lytic activity after repeated transplantations. For example, Hadfield, Magee, and Perry (19) observed that, after ten to thirty subcultures, some of their strains were decreasing in lytic potency. Of eleven active strains, they noted that six retained the same degree of activity during the period of study.

Observations also indicate that strains, with which the rate of reaction has slowed down, may be restored again to highly active ones both *in vitro* and *in vivo*. The factors which influence the yield of fibrinolysin by individual strains appear to be, in part, inherent in the bacterial cells and also to be related to the environments in which the organisms are kept viable. This

subject will be considered again further on. It is mentioned at this point to illustrate the fact that constancy in the yield of fibrinolysin has not been found to be a fixed attribute of all strains of hemolytic streptococci during periods of artificial cultivation.

The cultural factors which afford the most favorable basis upon which to make observations require: (a) Abundant growth. (b) Use of culture at time of maximum growth. (c) Use of culture media favorable for yield of fibrinolysin (possible influence of factors accessory to nutrition is suggested). It is important, also, to differentiate, in single tests on individual cultures, between strains that may, through prolonged laboratory cultivation or environmental circumstances, have become weakened in fibrinolysin production and other strains that are actually devoid of the property.

In the usual performance of the test, the plasma from the blood of *normal* human beings is regularly employed. However, the plasma-clots of different, apparently normal individuals may vary in susceptibility to lysis. For example, Tillett, Edwards, and Garner (66) noted that among thirty normal adults the plasma-clots from the blood of thirteen were liquefied within fifteen minutes, whereas the fibrin from eight others required from one to four hours before lysis was complete, even when a highly potent strain of hemolytic streptococcus was used in the tests. The dissolution time for the remaining nine normal persons ranged between fifteen minutes and one hour.

In addition to the clot available in whole oxalated plasma, fibrinogen and thrombin chemically isolated from blood have also served as a source of fibrin (65). The fibrin formed by combining fibrinogen and thrombin in the presence of active cultures has been found to liquefy at a greater speed and with smaller amounts of culture than does the clot of whole plasma. The probable explanations of the difference in speed of reaction between the substrates of whole plasma-clots and of fibrinogen-thrombin clots will be discussed in relation to immunological studies. From the standpoint of experimental procedure, the greater sensitivity of the fibrinogen-thrombin material has been



found to be useful in certain studies. However, it should be noted that additional complications may be introduced with the fibrinogen-thrombin technique. Investigators studying problems of blood coagulation have observed that when the fibrin, formed by relatively pure fibrinogen and thrombin, is allowed to stand for several hours, spontaneous lysis may occur with some preparations. Whether or not the spontaneous autolytic process may be catalyzed by the streptococcal fibrinolytic enzyme has not been studied. No information is available by which the end products of the autolytic and fibrinolytic actions may be compared. However, the possible effect of spontaneous lysis in fibrinolytic tests involving several hours' incubation may condition the evaluation of results obtained with fibrinogen-thrombin preparations, if the time required for lysis extends to several hours.

A final technical consideration concerns the influence of spontaneous retraction of the clot on the reading of the results of fibrinolytic tests. When active fibrinolysis occurs under the usual favorable conditions, the process is characteristic and the end point of the reaction is clearly defined. The quantities of materials employed in the usual test are such that, when coagulation occurs, the tube may be inverted without disturbing the position of the clot at the bottom. However, when the tubes are allowed to stand for a period of time, retraction of the clot may occur regardless of the presence or absence of bacterial cultures. The factors which determine the retractility of blood clots appear to be unknown. Consequently, neither the speed nor the degree of retraction is controlled in fibrinolytic tests. When the clot remains attached to the inner wall of the tube, it seems to occupy most of the space up to the top of the fluid level and it is saturated with liquid. Under these conditions, the reading of negative fibrinolysis is definite. However, when the clot is released from the sides of the tube, it settles to the bottom and may progressively shrink in size depending upon the degree to which the fluid contained within the interstices of the clot is squeezed out. An appearance comparable to the latter

incident may occur in fibrinolytic tests. In the experience of the writer, it may be difficult to differentiate weakly acting strains which have induced partial lysis after prolonged incubation from nonfibrinolytic strains in the tests of which a considerable degree of spontaneous retraction has occurred without lysis. Consequently some degree of reservation is indicated in the exact classification of cultures when the results are not clearly defined. It seems likely that a correct interpretation of some of the doubtful tests requires a method more accurate than the visual estimation of lysis on the basis of the size or shape of the ball of fibrin.

In performing the tests, the greatest number of observations have been made by incubating the preparations in the water bath at 37°C. Reports have indicated that incubation at higher temperatures may be preferable. Hadfield, Magee, and Perry (19) allowed the tubes to stand at 37°C. until coagulation had occurred. Following clot formation, 52°C. was used. They believed that lysis was hastened at the higher temperature. In some of his experiments, Schmidt (57) considered that more satisfactory results were obtained at 45 than at 37°C. Sherman and Niven (59) have advocated incubation at 53°C., after coagulation has occurred at room temperature. They pointed out that this procedure eliminated the growth of the organisms during the test period so that the result of the test was dependent upon the amount of preformed fibrinolysin. They noted that the duration of the tests could be shortened, since, if no lysis occurred in four hours, the result was not altered by prolonged incubation. Garner and Tillett (15) found that the reaction proceeded at a slower rate at room temperature than at 37°C., and that lysis was even more retarded at ice box temperature.

In summarizing the data concerning the materials and methods, emphasis has been placed upon factors which may affect the results obtained in fibrinolytic tests. Inasmuch as the phenomenon is readily demonstrable, the possible importance of some of the conditions may appear to have been unduly stressed. However, a review of some of the technical details

indicates the possible significance of experimental procedures in interpreting the results to be reported, in some of which discrepancies may be referable to materials and methods.

## II. THE TYPES AND KINDS OF BACTERIA, PARTICULARLY STREPTOCOCCI, WHICH POSSESS FIBRINOLYTIC ACTIVITY

The first positive tests of fibrinolytic activity were obtained with strains of hemolytic streptococci derived from patients suffering from acute illnesses. Additional information concerning the fibrin-dissolving action among many strains of streptococci has accumulated from the published reports of several investigators. The incidence of the fibrinolytic property has been considered in relation to certain individual and group characteristics of the organisms and also to other biological reactions of streptococci. Other species of bacteria have also been tested for the presence of lytic properties. Although in most instances the results have been negative, certain interesting findings have been reported.

### *a. Streptococcus hemolyticus of the beta type*

The first series of articles to be summarized under this heading deal with the results of fibrinolytic tests performed with hemolytic streptococci which were described by the authors as being associated with infections of varied clinical manifestations and degrees of severity. The details of the association have not been given in every instance nor is the correlation between the culture tested in the laboratory and the etiological status clearly established with many of the strains. However, the findings illustrate the occurrence of fibrinolytic properties among human pathogenic strains.

Tillett and Garner (65) tested the fibrinolytic activity of twenty-eight strains from different conditions including septicemia, acute tonsillitis, scarlet fever, erysipelas, empyema, and cellulitis. All of the strains were actively fibrinolytic. *Result*: 28 strains; 28 positive.

Hadfield, Magee, and Perry (19) reported the results obtained with twenty-nine strains that were derived from cases of moderate and severe scarlet fever, fatal septicemias, puerperal sepsis, peritonitis, tonsil-

litis, and rheumatic fever. The strains all caused lysis with varying degrees of potency and completeness during the test period. Five of them were highly active, six were somewhat less active, and eighteen produced slow or partial lysis. The highly active ones were from the severe cases. *Result:* 29 strains; 29 positive, with 11 highly active and 18 weakly lytic.

Madison (31) recorded the results obtained with thirty-two strains from "internal human tissues" and 123 from "superficial human tissues". The first group consisted of strains from cases of pneumonia, septicemia, empyema, and meningitis. Thirty of these were fibrinolytic. Of the 123 strains in the second group, which came from erysipelas, furunculosis, fistula, sore throat, sinusitis, and acute gastritis, only twenty-one were fibrinolytic. Five strains from erysipelas were highly potent. *Result:* 1st group, 32 strains; 30 positive (94 per cent). 2nd group, 123 strains; 21 positive (17 per cent).

Morales-Otero and Pomales-Lebron (43 to 45) in separate communications cite their results with thirty-three, fifteen, and forty-eight strains, respectively. The first and third groups were derived from a variety of disease sources. Of these eighty-one strains, seventy-nine exhibited the capacity to dissolve fibrin. (One of the negative strains came from a patient convalescent from scarlet fever; the other from a case of lymphangitis.) The group of fifteen strains were obtained from cases of recurrent tropical lymphangitis. Two of the strains were described by the authors as effecting incomplete hemolysis and appear not to have been of the beta type. The remaining thirteen strains were fibrinolytic. *Result:* 94 strains; 92 positive (98 per cent).

Hare and Colebrook (20), in one of their articles concerning infections due to hemolytic streptococci in parturient women, described the biological characteristics of a large number of strains. Of fifty-six derived from cases of puerperal infection, fifty-five were actively fibrinolytic. From eleven of the cases which had low-grade fever during puerperium, the strains in three instances were fibrinolytic. In some of these mild cases the authors considered that the fever was of uncertain origin. *Result:* 56 strains from puerperal fever; 55 positive (98 per cent). 11 strains from mild febrile puerperium; 3 positive (27 per cent).

Dack, Woolpert, and Hoyne observed the lytic action of 303 strains from scarlet fever. Twenty-five of them were derived from infected mastoids, and were all fibrinolytic. Of the remaining 278 strains, only twenty-eight caused lysis of fibrin. *Result:* 25 strains from scarlet

fever complicated by mastoiditis; 25 positive. 278 strains from scarlet fever; 28 positive.

Fraser and Madison (12) tested sixty strains from scarlet fever and found them all to be fibrinolytic. The highest potency was most frequent in the strains from severe cases. *Result*: 60 strains from scarlet fever; 60 positive.

Tillett (67) reported the results obtained with 157 strains. Of these, 140 were grouped, according to the source, into those from septicemia, acute suppurative diseases, (such as meningitis, peritonitis, empyema, mastoiditis, etc.), erysipelas, acute tonsillitis with and without rheumatic fever or nephritis, and a single additional group including chronic disorders and normal carriers. In these observations, the tests were made with the first subculture of the organisms after isolation from the patient. Of the 140 strains of definite etiological significance, 139 were fibrinolytic. An additional group of seventeen human pathogenic strains, obtained from other laboratories, were found to be actively lytic. *Result*: 157 strains from various disease sources; 154 positive (98 per cent).

Kodama (26) studied the biological properties of a large number of strains. Of 130 strains recently isolated from human infections and from the throats of normal people, 128 were fibrinolytic. *Result*: 130 strains; 128 positive (98 per cent).

Stewart (62) observed the lytic activity of 211 strains which produced soluble hemolysin. Of these, 146 were from surgical sources including puerperal infection, forty-five were from scarlet fever, and twenty from removed tonsils. One hundred and eighty-six of the total were classified as fibrinolytic. Of the twenty-five negative strains, sixteen were from surgical sources, seven from scarlet fever, and two from removed tonsils. The negative strains were tested on the first subculture. *Result*: 211 strains from various sources; 186 positive (88 per cent).

Evans (11) in a report on the properties of *Streptococcus pyogenes* cited the fibrinolytic properties of thirty-three strains. Thirty-two were fibrinolytic. In a subsequent article on *Streptococcus scarlatinae*, thirteen strains were tested. The average potency of the strains was not great, and four were found to be negative. Evans designated as *Streptococcus scarlatinae* strains which exhibited certain selective sugar fermentations, the most important of which was inability to ferment salicin. *Result*: 33 strains of *Streptococcus pyogenes*; 32 positive (97 per cent). 13 strains of *Streptococcus scarlatinae*; 9 positive (69 per cent).

Tunncliffe (71) studied, among several groups of streptococci, the occurrence of lysis by nineteen which were of the hemolytic type. They were isolated from scarlet fever, erysipelas, septic sore throat, endocarditis, and septicemia. All were actively fibrinolytic. *Result*: 19 strains; 19 positive.

Summarizing the findings just given, the figures are: Total number of strains, 1299, of which 899 (69 per cent) were actively fibrinolytic.

In the greatest number of the reports, however, the incidence of fibrinolytic activity by the pathogenic strains was greater than 90 per cent. Madison (31) in the tests with strains described as obtained from superficial human tissues, and Dack, Woolpert, and Hoyne (4) in their scarlet fever strains reported the lowest incidence (17 and 16 per cent respectively) of lytic properties. It may be noted that Evans (11) also considered *Streptococcus scarlatinae* to be less actively fibrinolytic than the *Strep. pyogenes* group. All of the reports with respect to *Strep. scarlatinae* strains have, however, not been consistent. Dack and co-authors considered the latter strains from severe cases to be highly potent.

The findings have demonstrated that strains from suppurative and invasive types of infection are, with few exceptions, not only regularly possessed of fibrinolytic properties, but are also usually the most potent in causing lysis of fibrin. For example, cultures derived from cases of septicemia, peritonitis, meningitis, or infections of the throat (acute tonsillitis, scarlet fever) where the organisms have invaded beyond the local pharyngeal tissues, constitute the strains which elaborate fibrinolysin in considerable quantity. The findings with cultures from minor infections and perhaps with some *Strep. scarlatinae* strains, indicate either the absence of lytic properties or that the production of fibrinolysin is characteristically impaired during laboratory cultivation. The suggestion, implied in these results, of a possible association between lytic activity and pathogenicity will be discussed later.

The occurrence of fibrinolytic properties in strains derived from normal persons has been studied less extensively. In twenty-five strains isolated from the throats of patients with various chronic disorders and from normal persons, Tillett (67) found the

incidence of weakly lytic strains to be greater than that of highly active ones. More detailed findings with cultures from normal throats will be given in association with the studies of relations to the Lancefield groups.

From the standpoint of the classification of hemolytic streptococci on the basis of biological, biochemical and serological reactions, the admirable and detailed review of Sherman (58) includes data concerning fibrinolytic activity of strains in relation to other findings. It would be repetitious to record here the reports which he has analyzed. Consequently, the reader is referred to Dr. Sherman's article for comprehensive data.

The fundamental observations of Lancefield (28) concerning the serological classification of hemolytic streptococci has had such wide and important application in the orientation of this species of organisms that it is of paramount importance in the study of strains. Sherman has brought together various findings concerning streptococci under the Lancefield groupings. Consequently the results given here will be limited to the fibrinolytic activity of strains with respect to the Lancefield classification.

#### *b. Relation to Lancefield serological classification*

Group A hemolytic streptococci have come to be recognized as the group characteristically responsible for acute infections in man. The fibrinolytic activity of strains identified serologically as belonging to Group A has been described in several articles with uniform results. Hare (21) reported on sixty-three strains from the nose and throat of normal persons, and found sixty-two possessed fibrinolytic properties. Kodama tested 160 Group A strains from cases of infections, from normal persons, and from stock cultures; and 157 were fibrinolytic. Davis and Guzdar found each of twenty-eight Group A strains from normal throats to possess lytic properties. Sherman and Niven reported four out of five strains which dissolved fibrin. They cite one strain, originally isolated from a case of epidemic sore throat which belonged to Group A but was nonfibrinolytic when tested. Hare and Maxted isolated ten Group A strains from the stools of patients with scarlet fever; each culture was active against fibrin.

Seegal, Heller, and Jablonowitz recovered from monkeys nineteen Group A strains and found them fibrinolytic.

Of the 285 strains identified as belonging to Group A by the investigators who tested them against fibrin, 280 (98 per cent) possessed fibrinolytic activity. Madison (32) suggested "a possible genetic linkage between these two specific bacterial characters." From observations upon 189 strains, he reported that the titre of fibrinolysin and the titre of Group A carbohydrate as determined by the ring test were closely correlated. However, as will be described in the reports which follow, strains other than Group A have been found to be fibrinolytic.

*Groups B, D, E, F, and H.* Without extending the details, strains belonging to these groups have been found to be negative by Hare, Hare and Maxted, Kodama, Sherman and Niven, and Seegal, Heller, and Jablonowitz. The reports include forty-one strains of Group B, fifty-four of Group D, a small number of Group E, eighteen of Group F and ten of Group H. Sherman and Niven have recorded some of their results as  $\pm$ , indicating that possibly a slowly acting lysis may have occurred with some of the strains. The source of all of the strains was the throat, stools, or vagina of normal persons, or milk.

*Group C.* Among fifty-seven strains derived by several investigators (21, 26, 6, 45, 59) from throat, or vagina of normal persons or throat of monkeys (56), fifty-four were found to be fibrinolytic. Of eleven strains isolated from milk (59), none was fibrinolytic. In the biochemical tests of the strains isolated from normal persons, trehalose was fermented but not sorbitol. In the milk strains, Sherman and Niven observed that trehalose was unaffected, but sorbitol was fermented. Sherman has suggested, therefore, on the basis of this difference the terms "Human Group C" and "Animal Pyogenes Group C." It is interesting to note that many of the strains belonging to the "Human Group C" are fibrinolytic, but that the "Animal Group C" are negative. The strains belonging to Group C have only rarely (21, 58) been reported, up to the present time, as occurring in infections in man. They constitute, therefore, a group of fibrinolytic streptococci, which have not been considered significant in human infections,



although Hare refers to two strains originally isolated from cases of erysipelas.

Reich has described the transformation of a strain of hemolytic streptococcus, Group A, which by prolonged and repeated passage through rabbits lost the original serological classification and gave positive precipitin reactions first with Group C antiserum and then with Group E antiserum. Coincidentally the fibrinolytic activity was also lost. When, however, the strain was cultivated in repeated subcultures in broth, the original Group A reaction returned and fibrinolysis was again demonstrable. Gay and Clark (17), on the contrary, reported that a human strain "H", which had been passed through rabbits for nineteen years, belonged, at present, to Group A, and was capable of liquefying fibrin. Data with regard to the loss by a strain of fibrinolytic activity coincident with change in serological type are limited to the report of Reich. It is apparent that confirmation by the use of a large number of strains is necessary before the suggestive finding is established.

*Group G.* Of seventy-nine Group G strains, derived like the Group C strains from the throat, vagina, or stools of normal persons, seventy were fibrinolytic (21, 22, 26, 6, 45, 56).

From the data with respect to serological classification, strains belonging to Groups A, C, and G have been found to be fibrinolytic. Strains belonging to Groups B, D, E, F, and H have proved to be negative. Reports of a considerable number of other strains which are nonfibrinolytic will be reported in connection with animal strains. In these latter strains, however, the serological classification was not made.

Using the Lancefield classification for the identification of human strains both from patients and normal persons, the combined tests of serology and fibrinolysis demonstrate a correlation in 98 per cent of the tests with Group A strains. In combination with the observations made with strains derived from active infection but not classified serologically, the similarity of the two groups of findings is apparent.

As an arbitrary test for the separation of human pathogenic strains from innocuous ones, the determination of fibrinolysis

is a helpful procedure but is not necessarily conclusive in every instance. The large proportion of nonfibrinolytic strains among the serological groups has been found in Groups B, D, E, and F, which on the basis of previous experience with immunological and biochemical tests have been classified as nonpathogenic for man. Fry has reported three fatal cases of infection due to Group B hemolytic streptococci. The strains were without fibrinolytic activity. Fry described the special characteristics of the pathological anatomy which differed from the usual changes observed in fatal cases of hemolytic streptococcus infection, and discussed the possible significance of the disease picture from the standpoint of the qualities of the infecting organism.

In addition to the non-fibrinolytic Group B strains which caused infection in Fry's cases, limitations on the evaluation of negative strains as non-pathogenic are also exemplified by a few other exceptional strains which have possessed definite etiological significance in active infection and which have been tested under advantageous laboratory conditions but did not exhibit lytic properties.

From the standpoint of the interpretation of positive fibrinolytic tests as indicative of pathogenicity, restrictions in the significance of the results are based on reports that strains belonging to Groups C and G are only occasionally significant in human infections but are frequently fibrinolytic. It is interesting to note, however, that the fibrinolytic strains of Groups C and G have usually been derived from human sources. Sherman and Niven are of the opinion that some strains of various hemolytic species may induce the slow lysis of fibrin.

In contrast to the dissolving action of human pathogenic strains, Tillett and Garner reported that hemolytic streptococci from animal sources were usually incapable of liquefying the fibrin of human blood. These findings have been extended in several reports, although serological classification was not regularly reported. Since the factors pertaining to animal strains concern the source of the fibrin substrate as well as the origin and biological characteristics of the cultures, a consideration of this interesting phase of the subject is given in Section IV. The present

section continues with results obtained with other kinds of streptococci and other species of bacteria commonly associated with man.

*c. Streptococcus viridans*

Of this variety of streptococci, Tillett and Garner tested six strains and found each to be nonfibrinolytic. Madison (33) reported thirty-three strains as negative in fibrin-dissolving tests. The same author, even after using methods of concentrating fibrinolytic material, was unable to obtain lysis with green streptococci. Stewart recorded that thirty-three strains belonging either to the *Strep. viridans* or *Strep. anhemolyticus* type, were not active in the liquefaction of clot. Schmidt obtained no lysis with green streptococci. Laca and Porzecanski found strains of *Streptococcus viridans*, *Streptococcus fecalis*, and *Enterococcus* to be nonfibrinolytic. Tunnicliff (71) stated that strains of *Streptococcus viridans* were nonfibrinolytic but that some of them inhibited clot formation.

Neter and Witebsky (48) subsequently presented a series of reports on the fibrinolytic and anticoagulating action of several species of bacteria. Although the immediate purpose of this review is not concerned with the so-called anticoagulating action of organisms, the findings which are related to fibrinolysis warrant consideration. Neter and Witebsky reported that, when the bacteria were cultivated in 2 per cent glucose broth, some strains of the following species were fibrinolytic; *Streptococcus hemolyticus*, *Streptococcus viridans*, *Enterococcus*, *Pneumococcus*, *B. coli*, *B. lactis aerogenes*, *B. friedländeri*, *B. pyocyaneus*, and *B. proteus*. They concluded that "fibrinolysin production is not limited to hemolytic streptococci alone, if, for instance, the sugar content of the culture media is increased." If this reviewer understands the article correctly, tests for fibrinolytic activity were considered positive if clot formation failed to occur when  $\text{CaCl}_2$  was added to the mixtures of plasma and culture. Witebsky and Neter (81) also described what they considered to be the properties of two different fibrinolysins produced by streptococci. One of these had the following characteristics: It developed when the organisms were grown in 2 per cent glucose broth; it inhibited

clot formation; it was effective in both human and animal plasma; it acted only in an undiluted state; it was thermostable; and it was not neutralized by antistreptococcus sera. The other fibrinolysin was produced in 0.05 per cent glucose broth; it acted only upon human fibrin-clot; it was effective in high dilutions; it was thermostable, and was neutralized by antistreptococcus sera.

Witebsky and Neter stated that, when cultures of *Streptococcus viridans*, *Enterococcus*, or *Pneumococcus* were cultivated in 2 per cent glucose broth, fibrinolysin developed like that present in cultures of hemolytic streptococci also grown in 2 per cent glucose broth.

The inhibiting effect on clot formation exerted by cultures of streptococci had previously been noted by Dennis and Berberian and by Tunnicliff. In the latter studies, the culture medium of choice was, respectively, 2 per cent dextrose broth (9) and 1 per cent meat extract broth with 1 per cent dextrose (71).

Dart reported a confirmation of the findings of Neter and Witebsky with respect to fibrinolysin and anticoagulant (second fibrinolysin) if hemolytic streptococci were cultivated in 0.4 per cent dextrose broth. The fibrinolysin was obtained from cultures by precipitation with alcohol according to the method described by Garner and Tillett; the anticoagulant factor was recovered from the supernatant fluid by evaporation; it resisted heating at 100°C for 30 minutes.

Dennis and Adham in a further study of the anticlotting factor of dextrose-broth cultures of streptococci described it as being soluble in 75 per cent alcohol, absolute alcohol, and ether; it was dialyzable; it gave a strongly positive Kelling's test for lactic acid. They concluded that the anticoagulant was primarily lactic acid. The anticlotting constituent seldom occurred with cultures grown in media having less than 0.4 per cent dextrose; and the authors considered the anticlotting action to be more closely correlated with the total acid content of the cultures than with pH.

Tillett (69) studied the anticoagulating effect and the fibrinolytic activity of strains of *Streptococcus hemolyticus*, *Streptococcus viridans*, and *Pneumococcus*. The cultures were cultivated in 0.05, 1.0, and 2 per cent dextrose broth. With respect to the anti-

clotting effect, he found that when the ultimate pH of the 1.0 or 2.0 per cent dextrose-broth cultures was below 5.0, coagulation of plasma was inhibited; when the pH of the cultures was above 5.0, clotting occurred in all the tests but fibrinolysis was effected only with strains of *Streptococcus hemolyticus*. With uninoculated sterile broth of varying hydrogen ion concentrations, the effects on the coagulation of plasma paralleled the findings obtained with cultures of the same pH. Furthermore, when the high degree of acidity (pH 4.4 to 4.9) produced in dextrose broth cultures was altered by the addition of NaOH to pH 6.0 to 7.0, coagulation occurred. When cultures in 0.05 per cent dextrose broth (pH 6.5 to 7.0) were acidified to below pH 5.0, coagulation was inhibited. In studies on the physiology of blood coagulation, the lower limit of pH at which fibrin is formed is placed at 5.6 to 6.0. It is also interesting to note that the anticoagulative action of organic acids, including lactic acid, has been described (80). Tunnicliff and Hammond (72) in continuing a study of the ant clotting action of *Streptococcus viridans* found that the smooth form, which prevented coagulation, lowered the pH of 1 per cent dextrose broth to 4.4-4.8; cultures of rough forms, however, which did not inhibit coagulation, reached a pH of 5.2-6.0.

From a consideration of the data concerning the ant clotting action of various bacterial species, it seems probable that the effect depends to a considerable degree on the action on oxalated plasma of the products of the hydrolysis of sugar by the organism, or on pH, or on both of these factors. Furthermore, from an analysis of the findings with respect to organisms other than hemolytic streptococci, it appears that the action designated as fibrinolysis by *Streptococcus viridans*, pneumococci, and other bacterial species, is not due to a lytic agent comparable to the fibrin-dissolving substance of hemolytic streptococci.

#### d. Other streptococci; Dissociants

*Pseudo-hemolytic streptococci*. This term has been frequently employed by English investigators in designating strains which differ from other hemolytic streptococci on the basis of negative tests for "soluble hemolysin." Hare and Colebrook describe the

results of fibrinolytic tests with thirty-four such strains. None of them caused lysis of fibrin. Twenty-seven of the strains were from pregnant women who had afebrile puerperium. Seven came from puerperal cases with mild fever. Stewart (62) found that twenty-seven strains of the pseudo-hemolytic variety were nonfibrinolytic.

*Streptococcus anhemolyticus*. Only a few strains of this type have been tested. Tillett and Garner obtained negative results with two strains; and Stewart described anhemolytic strains as being negative in fibrinolytic tests.

*Double-zoned hemolytic streptococci*. Brown has described strains having this characteristic appearance when cultivated in blood agar. Strains of this type have been derived from both human and animal sources. The author has tested some of them, obtained through the courtesy of Dr. Brown, and found them to be nonfibrinolytic.

*Dissociants of streptococci*. Mellon and Cooper (42) described the action of various dissociants which they obtained from individual strains of hemolytic streptococci. Some of the dissociated forms were described as nonhemolytic diphtheroids. The variants, which caused only partial lysis in 24 hours, were definitely less active in liquefying fibrin than the original cultures. The authors also state that diphtheroids with acid-fast granules considered to be in the tubercle bacillus cycle were indistinguishable in their fibrinolytic activity from diphtheroids dissociated from streptococci. Subsequent reference will be made, in relation to virulence, to the findings of Tunncliffe (68), who noted the loss of lytic activity by certain strains associated with the change from cultures producing smooth colonies to those producing rough, irregular colonies, and also to the results obtained by Dawson and his coworkers (7), who, with M, S, and R variants of the same strain, observed no difference in fibrinolysis, each of the cultures being active.

#### *e. Staphylococcus and other bacterial species*

The effect of staphylococci on the coagulation of blood and the dissolution of fibrin has received the attention of many investigators. It is not within the scope of this review to consider these

properties of staphylococci, because the slowly liquefying action of staphylococci on fibrin, although constituting an example of bacterial fibrinolysis, differs in many respects from the rapid fibrin-dissolving effect of hemolytic streptococci. Madison (35) has described the immunological differences between the products of the two bacterial species.

Concerning other bacterial species, the available reports are limited. Tillett and Garner tested several members of the colony-typhoid group and also *Hemophilus influenzae* and found them to be nonfibrinolytic. Schmidt tested a heterogeneous group of organisms and obtained uniformly negative results. Madison (40), however, obtained interesting results with *B. pestis*. Sixteen strains were tested for fibrinolytic activity. One of them was of human origin (20 years old), and the others were derived from field mice and ground squirrels. For the fibrin clot, he used fibrinogen and thrombin obtained from the plasma of man, guinea pig, rabbit, rat, and other animal species. Using methods of titration which he described, Madison found that the cultures of *B. pestis* induced lysis of the fibrin-clots from the blood of several of the animal species, including man. The potency of lytic action was, however, greatest against the coagulum of rat's blood.

Fisher (11a) in studying the fibrinolytic properties of staphylococci noted that certain contaminating bacterial species dissolved plasma-clot slowly in one to six days. The strains consisted of *B. subtilis* (5 strains), and single cultures of *B. proteus*, *B. pyocaneus*, diphtheroids, and *B. alkaligenes*. Owing to the fact that an incubation period of several days was necessary before dissolution occurred, the possibility that the liquefaction might be dependent upon proteolytic digestion warrants consideration. No studies dealing with this point have been made.

Weiss (80a) made observations with two strains of *Bacterium melaninogenicum*. The cultural material was concentrated through alcoholic precipitation, and the tests were made with human fibrinogen-thrombin preparations. A 1 to 4 dilution of the concentrate of one strain caused lysis in forty minutes, while original concentrations of the other strain caused partial lysis (designated 2+).

Neter and Witebsky (48) found that *Pneumococcus* behaved like *Streptococcus viridans* in fibrinolytic studies with dextrose-broth cultures. Tillett and Garner, Schmidt, Lippard and Johnson, and others could demonstrate no fibrinolysis with pneumococci.

### III. CORRELATION OF FIBRINOLYTIC ACTIVITY WITH OTHER BIOLOGICAL PROPERTIES OF HEMOLYTIC STREPTOCOCCI

#### *a. Relation to proteolysis*

Laca and Porzecanski studied the proteolytic, fibrinolytic, and hemolytic activity of ninety-six strains of streptococci. They found all of these properties commonly present in many of the pathogenic strains. However, in certain strains, fibrinolysis was present but proteolysis was absent; while in others the proteolytic effect was marked, but fibrin dissolution did not occur. Garner and Tillett by determinations of amino nitrogen contrasted the action on fibrin of fibrinolysin and streptococcal peptase.

#### *b. Relation to the production of hemolysin and of toxin*

With respect to the qualitative differences of hemolysins of streptococci, since the relationship is contained in the reports listed under the kinds of streptococci classified on the basis of their action on blood agar, the results need not be restated. Among strains of hemolytic streptococci of the *beta* type, accurate comparative measurements of hemolysin and fibrinolysin have not been made. However, on the basis of the size of the zone of hemolysis created by colonies in blood agar, Hadfield and associates, Schmidt, and others have stated that no strict relationship exists between potency of strains in the production of hemolysin and of fibrinolysin.

Fraser and Madison using scarlatinal strains attempted to correlate fibrinolytic activity, toxin production, and severity of scarlet fever. They found a 63 per cent correlation between the titre of toxin produced by the strains and severity of disease graded according to degree of fever, duration, and complications. On the same basis they reported an 80 per cent correlation between the titre of fibrinolysin and severity of illness. They stated that their results agreed with the conclusions of Dack and



his associates that a high fibrinolytic titre is significant in relation to the complications of scarlet fever.

Morales-Otero and Pomales-Lebron (44) compared fibrinolytic activity with toxigenicity as determined by intracutaneous tests in the shaved skin of white goats. Of fifteen strains, thirteen were both toxigenic and fibrinolytic.

### *c. Relation to virulence*

The types of illnesses resulting from hemolytic streptococcus infections are characteristically diverse. The manifestations of the diseases range from clinical entities, the etiology of which may be diagnosed or suspected without laboratory aid, to other disorders which have characteristics common to many pyogenic infections. The mechanisms of hemolytic streptococcus infections appear to involve properties which are integral parts of the bacterial cell body, such as capsule formation, and perhaps others, and also substances which are elaborated and excreted by the organisms. That hemolytic streptococci produce different kinds of noxious agents is evidenced by many reports and is particularly well illustrated by the erythrogenic toxin and the hemolysin. These substances possess different properties and have been studied as separate entities, although elaborated by the same types of organism. Furthermore, with the possible exception of the studies of Mudd and his associates (3), the occurrence of the excretory products in strains has not, up to the present time, been found to parallel any individual constituent of the bacterial cell structure. Concerning the production of fibrinolysin by streptococci in relation to structural characteristics of the organisms, a few observations have been made. Hadfield, Magee, and Perry observed with two strains, which produced matt colonies (virulent) at the time of high fibrinolytic activity, that the subsequent change to cultures producing glossy colonies (avirulent) was attended with marked reduction in the production of fibrinolysin. They found the average virulence for mice of their strains most potent in the production of fibrinolysin was higher than that of the least active. Tunnicliff (71) reported that strongest lytic action was associated with virulent strains

which possessed capsules and produced smooth colonies. She found that the production of fibrinolysin was lost when cultures were altered by dissociation so that granular colonies with irregular edges were formed. She reported further that reversion of strains to the type which formed smooth colonies, was accompanied by the restoration of active fibrinolysis. Schmidt noted the loss of lytic activity with some strains after repeated subculture, and that virulence for mice was also lost. When, however, by mouse passage, virulence was restored, lytic action also increased.

Morales-Otero and Pomales-Lebron (44) cited their experience with strains which were virulent for mice at a time when the organisms were fibrinolytic. Two years later, the same strains had lost mouse virulence but had retained fibrinolytic and toxigenic powers. Dawson, Hobby, and Olmstead in describing the results of their extensive studies on M, S, and R variants of hemolytic streptococci briefly record, without giving details, that no significant differences were observed in the fibrinolytic capacity of the three variants of the same strain.

These findings indicate that although the production of fibrinolysin by hemolytic streptococci may frequently accompany the presence of experimental indices of pathogenicity (colonial structure and virulence for mice), the relationship is not an inseparable one. In the author's experience, strains of hemolytic streptococci of highest fibrinolytic potency may not be virulent for mice. Furthermore, as will be subsequently discussed, human strains of hemolytic streptococci are not regularly capable of causing dissolution of the fibrin of mouse's blood. Since the presumptive evidence of the rôle of fibrinolysin in virulence is derived from the capacity of the invading organism to dissolve the fibrin of the infected animal, it follows that invasion in the absence of fibrin susceptibility is referable to other conditions. In experimental infections, the mechanism of virulence often centers around factors which involve susceptibility or resistance to phagocytosis. These same factors, in all probability, play an important and often decisive rôle in human infections. However, in infections in man due to hemolytic streptococci, supple-

mentary factors may influence the pathogenesis of the diseases. For example, the erythrogenic toxin, which seems to be of limited significance in infections of laboratory animals, induces toxic manifestations in man. Whether or not the fibrinolytic properties of human pathogenic strains may also be a contributing factor to some of the characteristic elements of hemolytic streptococcus infections, has not been determined but may be surmised from suggestive indirect evidence.

Neter (50) found the fibrinolysin present in the spinal fluids of four out of five patients with meningitis due to hemolytic streptococci. With samples of the spinal fluids of cases of meningitis due to other organisms, he obtained negative results, except in one case of pneumococcus meningitis. He also reported the occurrence of lytic activity in peritoneal and pleural exudates from hemolytic streptococcus infections, and with the pericardial and peritoneal fluids from *Staphylococcus aureus* infections. He examined also the peritoneal exudate of mice infected with hemolytic streptococci and pneumococci. In the infections with streptococci, the peritoneal washings induced lysis of human fibrin, but the material from mice infected with pneumococci was negative.

In connection with the production *in vivo* of fibrinolysin, it may also be mentioned that the thinness of the fluid so characteristic of the exudate obtained early in cases of infections of the serous cavities due to hemolytic streptococci, particularly empyema, appears to be due to the lytic action of the infecting organisms on the fibrinous exudate. Goodpasture in describing the pathological changes occurring in bronchopneumonia due to hemolytic streptococci of 1917-18 refers to cases in which "microscopically the alveoli are filled with polymorphonuclear leukocytes and usually enormous numbers of streptococci, with little or no fibrin." In MacCallum's account of pneumonia during the World War, reference was not infrequently made to areas in which fibrin was scarce or absent. The density of fibrin deposits in many of the lesions was also commented upon. The present writer has examined material from two cases of empyema. Fibrinolysin was demonstrable in the thin pleural fluid obtained early in the disease. However, as the exudate became thick with

fibrin, antifibrinolytic properties were demonstrable in the blood of the patients. It seems not unlikely that the pathogenesis of some aspects of hemolytic streptococcus infections may be explained on the basis of the fibrinolytic potency of the organism in relation to the antifibrinolytic properties of the host.

#### IV. HEMOLYTIC STREPTOCOCCI FROM ANIMAL SOURCES, WITH PARTICULAR REFERENCE TO ACTION ON FIBRIN OF BLOOD FROM DIFFERENT ANIMAL SPECIES

Tillett and Garner reported that, although cultures of hemolytic streptococci derived from patients caused lysis of normal human fibrin-clot, normal rabbit fibrin-clot was resistant to dissolution when tested under comparable conditions. Observations concerning differences in fibrinolytic activity referable to animal sources of fibrin have yielded interesting results.

Van Deventer and Reich (73) tested three human strains and two animal strains (P 454 and K 158 E of Lancefield) against the plasma-clot of the following animals: rabbit, guinea pig, rat, domestic fowl, horse, cow, goat, sheep, dog, and cat. All tests were negative. The three human strains were lytic for human fibrin. They were also tested against the plasma-clot of rhesus monkeys. Two of the strains caused lysis of monkey fibrin but at a slower rate than the effect on human fibrin. One of them was equally active against human and monkey fibrin.

Madison (34) tested twelve strains of hemolytic streptococci derived from horses suffering from strangles against samples of fibrin derived from horse, man, hog, cow, and rabbit. Fibrinogen-thrombin preparations were employed because of their greater susceptibility to lytic action. The equine strains caused dissolution of horse fibrin but did not liquefy the fibrins derived from the other animal species, including man. Two human strains of hemolytic streptococci were weakly lytic against *horse* fibrin. In addition, Madison found that three strains of hemolytic streptococci obtained from hogs (septicemia) were highly active against *hog* fibrin. The same strains were weakly active against *human* fibrin, but negative against the fibrin of the other animal species.

Planet also compared the action of *human* and *equine* strains of

hemolytic streptococci against the plasma-fibrin of *human* and *equine* sources. The single *human* strain of hemolytic streptococcus, which he employed, caused dissolution of the fibrin from five different *human* plasmas, but was inactive against the fibrin of twenty-two different *horse* plasmas. One of his *equine* strains caused lysis of all of the samples of fibrin from *horses* but was negative against *human* fibrin-clot. With other *equine* strains of hemolytic streptococci, varying degrees of lytic activity for *equine* fibrin were noted but no alteration of human fibrin occurred. Some of the equine strains fermented lactose and some did not. No relationship was noted between the fermenting activity and fibrinolytic capacity.

Smith, Hankinson, and Mudge tested twenty-two strains of hemolytic streptococci derived from *cow's milk* against the plasma-clot of *bovine* blood. Nine of the strains caused varying degrees of lysis of *bovine* fibrin. Of these, two were from normal cows, five were from cows with mastitis in a quarter other than that which supplied the infected milk, and two were from cows with chronic mastitis. The results were not conclusive, but suggested the possibility that strains lytic for bovine fibrin might be significant in mastitis.

Pilot, Buck, and Davis (53) examined one hundred strains of hemolytic streptococci obtained from the tonsils of cows; and ninety-two gave negative fibrinolytic tests with human fibrin. Among forty-three strains derived from the tonsils of hogs, thirty-nine were negative. No report was made of tests made with fibrin of the cow or hog. In a subsequent article twenty-two canine strains were reported as negative for human fibrin (54).

Seegal, Heller, and Jablonowitz in a study of hemolytic streptococci derived from *monkeys*, tested the fibrinolytic activity of the cultures against fibrin from *man* and from *monkeys*. Nineteen Group A strains caused lysis of *human* fibrin within  $3\frac{1}{2}$  hours, and also dissolved *monkey* fibrin but at a slower rate, ranging from 6 hours with 3 strains to a negative result with 2 others. With four Group C and five Group G strains, *human* fibrin was liquefied regularly within  $3\frac{1}{2}$  hours, and lysis of *monkey* fibrin occurred with the same prolonged rate of activity obtained with

the Group A strains. The lysis of *human* fibrin was uniformly more efficient than that of *monkey* fibrin.

Yen, in studying the problem of the resistance of animal fibrins to dissolution, observed the influence of quantitative factors in the reaction. Using hemolytic streptococci from patients, he concentrated the fibrinolysin from filtrates of cultures by alcoholic precipitation. In order to have a more sensitive substrate, he employed fibrinogen-thrombin preparations isolated from the plasma of man, rabbit, and guinea pig. He found that human fibrin was dissolved in 3 to 5 minutes, that rabbit fibrin was liquefied in 30 to 180 minutes, and that guinea pig fibrin failed to liquefy. He concluded that rabbit fibrin-clot was not absolutely resistant to lysis by human strains of hemolytic streptococci, if sufficiently high concentrations of fibrinolysin were tested.

Schmidt also emphasized the importance of the quantitative factor in determining the results obtained with materials from different animal species. Although he found exceptions with some strains, he confirmed the findings of others with respect to the homologous source of materials, *provided* the usual test dose of culture was used and the fibrin was contained in the clot of whole plasma. When, however, large amounts of fibrinolysin were employed and added to fibrinogen-thrombin preparations, the principle of species specificity was not regularly maintained.

Concerning the sensitivity of the fibrin substrate to dissolution by streptococci, an additional complicating factor is introduced when the fibrinogen constituent of the clot and the thrombin component are each derived from a different animal species. Tillett and Garner reported that when fibrinogen from rabbit's blood was coagulated in the presence of cultures of hemolytic streptococci by thrombin from human blood, dissolution occurred; also, when fibrinogen of human blood was clotted by thrombin of rabbit's blood, liquefaction took place. When, however, both constituents of the coagulum were derived from the rabbit, the results were either negative or slow dissolution occurred after many hours. In the above experiments, the determining factor in the occurrence of active fibrinolysis was the

presence of at least one human element in the fibrinogen-thrombin complex.

Madison (36) used materials which he designated as "hybrid fibrins." He derived fibrinogen and thrombin from eleven different animal species, including man. Using a human strain of hemolytic streptococcus, he found that dissolution occurred in every instance when the fibrinogen component of the fibrin was of human derivation regardless of the source of the thrombin. When the human component was thrombin and the fibrinogens were from various animals, dissolution occurred, but proceeded at a slower rate than the control of human fibrin. If neither constituent was of human origin, the results were negative. Comparable but somewhat less striking homologous species relationships were found to exist when an equine strain active against horse fibrin, and a porcine strain active against hog fibrin, were tested with hybrid fibrins. In these latter experiments, however, there were some irregularities not explicable on the basis of the individual animal source of the materials.

The subject of hybrid fibrins is obviously a somewhat confused one. Schmidt emphasized the importance of the quantitative proportions between fibrinolysin and fibrin substrate. He found that small doses of a highly active human strain acted only upon fibrins when one element was of human origin. When the amount of fibrinolysin was increased, however, some of the hybrid fibrins were dissolved. Schmidt extended the studies by considering whether or not strains, which are highly pathogenic for a given species, would dissolve the fibrin of the species provided thrombin of the homologous animal was employed. His results were not harmonious. They conformed to a homologous species relationship between virulence and source of fibrin with some strains, but the correlation was not demonstrable with others. For example, he described an equine strain, virulent for mice, which liquefied mouse fibrin formed with horse thrombin, but was inactive against mouse fibrin formed with human thrombin.

It is obviously impossible in the present state of knowledge to interpret clearly the results obtained with the manifold hybrid fibrins. It seems probable that the results are dependent upon

quantitative factors in some instances, and upon qualitative differences of materials in others. However, even when the differences are quantitative, homologous fibrin has been found to be more susceptible than heterologous material. Viewed as a chemical reaction involving a system consisting of enzyme (fibrinolysin) and substrate (fibrin), variations in sensitivity are dependent upon the sources of materials, but the degree of specificity necessary to elicit the dissolving effect is not established. Furthermore, since the materials used are not chemically pure, accessory factors, which may influence enzyme systems such as the fibrinolytic process, merit consideration. Additional information on this complex subject seems to require chemical procedures which are more technically exact than the methods employed at present.

In spite of the limitations on the interpretation of the results just discussed, the apparent predilection of strains of hemolytic streptococci for the fibrin of a species homologous to that in which the organisms may survive, and in some instances invade, contains implications of biological interest which invite additional study.

#### V. CHARACTERIZATION OF FIBRINOLYSIN AND NATURE OF THE REACTION

The fibrinolysin has been found to be freely excreted by the living, growing bacterial cells. Consequently, it has been possible to obtain active fibrinolytic material, free from the microorganisms, by filtration. Garner and Tillett found that the fibrinolytic principle could be partially purified by (a) precipitation of culture filtrate with 3 volumes of 95 per cent alcohol, and, (b) adsorption on polyaluminum hydroxide B of Willstätter followed by elution with  $M/10$  sodium phosphate buffer, pH 7.3. Concentration was accomplished by dissolution of the precipitates in small quantities of solvent, but was best obtained by vacuum dialysis (15). Concentration by alcoholic precipitation has also been reported by Madison (33), Yen, and Schmidt.

It should be noted that when high degrees of concentration are attempted, preparations may be encountered, which inhibit



coagulation. The explanation of the anticoagulative effect is not clear. It seems possible that it may be referable to some other constituent of the filtrate which is also concentrated together with the fibrinolysin. For example, peptone is known to contain anticoagulating material, which might be responsible for the effect. It seems also possible that inhibition of the clotting process might be dependent upon the physico-chemical action of highly concentrated proteins or other organic materials.

Garner and Tillett found that active culture filtrates were relatively heat stable, in some instances resisting heat of 100°C. for 60 minutes. Dennis and Berberian (9) reported that fibrinolytic activity was markedly weakened by boiling for one-half hour. In contrast to the heat stability of culture filtrates, Garner and Tillett observed the activity of material obtained by alcoholic precipitation was destroyed at 57°C. for one hour. However, when the fibrinolytic agent was purified by adsorption and elution, the resultant material was again heat-stable as in the case of the culture filtrate. The sensitivity of the material obtained by alcoholic precipitation suggests that the procedure separated the active principle from other substances which afforded protection from the deleterious effects of heat. Although an explanation of the differences in the effect of heat is not clear, the thermal properties suggest that the fibrinolysins of different preparations may exhibit variations in sensitivity to other inactivating substances, such as chemicals or antisera.

The fibrinolysin conforms in many of its characteristics to a protein. The partially purified materials give positive tests for protein, and fibrinolytic activity is destroyed by digestion with trypsin or papain (15).

Using fibrinogen-thrombin preparations, Garner and Tillett found that the fibrinolysin was not bound to the reaction products, since the active material was recovered approximately quantitatively after dissolution of fibrin was complete.

In characterizing the fibrinolysin, therefore, on the basis of the data available at present, the active agent may be considered to be enzymic in nature for the following reasons: 1. It is of biological origin. 2. Catalytic property is indicated by the fact

that active material is recoverable, approximately quantitatively, after the reaction is completed. 3. Destruction by heat (high temperatures for broth filtrate; low temperature for material isolated by alcoholic precipitation). 4. Tests for protein are positive.

The fibrinolysin differs, however, from proteolytic enzymes in that preparations of the former exert no hydrolytic action on casein, gelatin, or peptone. Furthermore, it also differs from the so-called streptococcal peptase, which is obtained by rupturing the bacterial cells and which acts upon casein but is especially vigorous against peptone (15).

Fibrinogen is the only substrate besides fibrin which has so far been found to be susceptible to fibrinolysin. Demonstration of the action on *human* fibrinogen was made in experiments (15) in which fibrinogen, incubated for short periods with fibrinolytic cultures, was incapable of forming fibrin following the subsequent addition of thrombin. *Rabbit* fibrinogen, however, in parallel experiments, retained the capacity to form fibrin even after preliminary incubation of eighteen hours with fibrinolysin.

One of the interesting features of the fibrinolytic phenomenon concerns the nature of the end products of the reaction. Following dissolution of fibrin and during subsequent incubation, determinations have been made of increases in amino N (Garner and Tillett), and also of non-protein N and of the evolution of ammonia (Garner). It was found that, during the experimental period, there is a small and gradual increase in the amino N content of the solution. The results contrast, however, in degree very markedly with the observed effect of trypsin on fibrinogen, where the sharp increase in amino N, characteristic of proteolytic fermentation, occurred. Whether or not the action of fibrinolysin is accompanied by proteolytic hydrolysis, is not clear. The end products appear to be protein but to have somewhat different properties from fibrinogen with respect to thermal precipitation point and the precipitating concentration of salts. Garner did not detect the evolution of ammonia during the experimental period.

From these experiments it seems likely that the chemical deg-

radiation of the highly complex molecules of fibrin is not great, even though the physical change of solid fibrin into a solution is striking.

In referring to the observations of Garner and Tillett, Jablonowitz calls attention to the fact that globulin present in the impure preparations of fibrinogen may have accounted for the properties of the end products of the reaction rather than a change in the characteristics of fibrinogen to globulin through the action of fibrinolysin. Jablonowitz studied the alterations in the immunological specificity of fibrinogen following the action of fibrinolysin derived from a strain of hemolytic streptococcus of human origin. For purposes of obtaining highly purified material, he prepared fibrinogen by methods of repeated precipitation. This material, when tested against the antiglobulin serum described by Kendall, gave only a very faint reaction. Consequently it was used in the immunization of rabbits. The sera of the immunized rabbits was tested against two preparations: (a) sterile broth + fibrinogen, (b) fibrinolysin + fibrinogen. The two mixtures (a and b) were incubated for 24 hours at 37°C. before being used in precipitation tests with antifibrinogen serum. After the precipitation tests had been incubated, the precipitates were centrifuged, washed, and analyzed for total N. The total N in the precipitate produced with fibrinolysin + fibrinogen (b) was less (0.075 mgm.) than that obtained from the sterile broth + fibrinogen mixture (0.31 mgm.). Jablonowitz concluded therefore that fibrinogen was altered immunologically by the action of fibrinolysin. In other experiments to determine the rate of alteration, he found that there was an initial lag period of approximately 15 minutes followed by a rapid change which seemed to be complete in about an hour.

Garner (16) reported that the end product was not differentiated from fibrinogen by serological reactions. The findings, on which that observation was based, were obtained by Garner and Tillett (unpublished) in determining the precipitative titre by the usual technique. Using progressive dilutions of precipitinogen, the differences in the end points of the tests with fibrinogen and dissolved fibrin were not sufficiently great to indicate differences in the precipitinogenic preparations.

Doudoroff investigated the effect on fibrinolytic filtrate of cultures of various bacterial species. After mixing 48 hour cultures with the filtrate, he subsequently killed with chloroform the organisms which had been added and tested the mixture for fibrinolytic action. He found that the fibrinolysin was most regularly inactivated by bacteria which were capable of liquefying gelatin. The inactivating effect of the cultures was usually destroyed by heating at 60°C. for 30 minutes.

Madison and Snow (41) tested the antifibrinolytic effect of several antiseptics which they employed in sub-bacteriostatic doses in cultures. The results were not striking. They also added antiseptics to fibrinolytic tests and concluded that tincture of iodine impaired lytic action more definitely than other drugs.

Huntington cultivated strains of hemolytic streptococci in 0.05 per cent glucose-broth with and without 20 mg. per cent of sulfanilamide, and was unable to observe any deleterious effect upon the production of fibrinolysin by the drug.

## VI. IMMUNOLOGICAL STUDIES

In immunological studies, oxalated plasma from the blood of normal individuals and patients has been most regularly employed. By this procedure, the measure of antifibrinolytic resistance is made with the fibrin of the patient's blood in the presence of whatever antifibrinolytic properties may be concomitantly contained in the additional constituents of the same sample of plasma. Serum has also been employed as in other immunological reactions. However, owing to special conditions of the tests, which will be referred to later, the serological method has not been regularly adopted.

Although 0.2 cc. of plasma has been usually employed, inquiry has been made into the possible significance of differences in the amount of fibrinogen contained in blood in different diseases. Hadfield and associates investigated this point and found that the content of fibrin in plasma did not appreciably affect the dissolution time, even when as much as 1400 mgm. per 100 cc. of blood was present. Van Deventer (75) concentrated fibrinogen fourfold and found that the speed of dissolution was slowed but did not result in complete refractoriness. From these find-

ings, it seems unlikely that, under the condition of usual tests, significant variations in the dissolution time are referable to the quantities of fibrin in the blood.

The value of using strains of hemolytic streptococci of highly potent fibrinolytic activity in antifibrinolytic tests has been advocated by investigators of the subject. In order to emphasize the difference between the results obtained with normal susceptible fibrin and patients' resistant plasma-clot, Tillett, Edwards, and Garner (66) employed the whole broth culture of a strain of maximum potency. By this procedure the greatest amount of fibrinolysin was contained in the test material, including such additional amounts as the living organisms might produce during the period of incubation.

Hadfield and his co-workers considered the use of a powerfully lytic strain important in differentiating between the rate of dissolution of normal fibrin and of that from patients. Stuart-Harris (63), using data derived from titration experiments, illustrated graphically the characteristic curve of the relationship between concentration of lytic agent and time required for fibrinolysis. On the basis of the ratios obtained, he concluded that the use of weakly active strains or high dilutions of potent strains so prolonged the dissolution time with normal fibrin that the assay of the degree of resistance in patients' fibrin was masked. Furthermore, differences between samples of fibrin, which were minor when potent material was used, were unduly emphasized when weakly acting preparations were employed. Other observers have employed three to five strains in each test and used the average results.

Limited consideration has been given to the possibility that the fibrinolysins of different disease-producing strains may be immunologically distinct. Tillett, Edwards, and Garner tested the blood of a few patients with the homologous strain derived from each patient but were unable to detect any difference in antifibrinolytic resistance. Van Deventer (74) tested forty strains against the fibrin of three normal persons and two resistant patients. He concluded that there was only one type of fibrinolysin among the strains. Yü and Zia described their findings with

plasma from a patient convalescent from scarlet fever which was shown to be resistant to a strain from a case of puerperal sepsis, but susceptible when tested with some of the scarlet fever strains. They did not clearly indicate whether all of the test strains possessed the same degree of fibrinolytic potency. At the present time, among human strains no definite evidence of immunological differences of the fibrinolysins has been obtained, although an exhaustive study of the subject has not been made.

Determinations of the presence or absence of resistance have been made by contrasting the brief length of time required to liquefy normal fibrin with either the absence of any dissolving effect on patients' fibrin or the prolonged period necessary to effect liquefaction. The three variables in fibrinolytic tests are: quantity of fibrin; quantity of fibrinolysin; time required for dissolution. Fibrinolytic "units" have been suggested by some observers. Madison and Taranik (39) proposed that the highest serial dilution of broth culture causing complete liquefaction of the fibrinogen-thrombin clot by the end of two hours incubation be assumed to contain one fibrinolytic unit. From the dilution, the number of lytic units per cubic centimeter of broth culture was calculated. Van Deventer (76) referred to a unit of fibrinolysin as three times the amount necessary to dissolve, within two hours, the fibrin of fibrinogen-thrombin preparations. Standards, however, have not been used extensively enough in studies of antifibrinolysin to be evaluated. A sufficient amount of information is not yet available concerning methods of quantitative measurement and the mechanism of the reaction to make improved procedures practicable. Consequently, estimations of resistance based on the factor of time has been most widely used. From the standpoint of exact quantitative measurements, the limit of experimental error is in all probability relatively broad. For this reason, rates of dissolution which might serve as sharp dividing line between normal and abnormal results have not been advocated. In the absence of arbitrary standards, most observers have, with minor variations, employed the following scheme for estimating degrees of resistance, when the amount of culture and plasma were kept constant: Dissolution

in less than one hour indicates susceptibility; dissolution in one to three hours indicates doubtful to weak resistance; dissolution requiring three hours or longer up to twenty-four hours indicates "definite" or "marked" or "partial" resistance; no dissolution during the twenty-four hour period of the test indicates "maximum" resistance. When several tests are set up with constant quantities of the same samples of plasma and culture, the dissolution time is constant within a narrow range of variation. Consequently, when the difference in time of liquefaction of two separate specimens of blood is a matter of several hours, the delayed rate assumes significance.

Concerning the susceptibility of the fibrin from normal persons, a sufficient amount of information has accumulated to indicate the average findings among healthy adults. Among thirty normal individuals, Tillett, Edwards, and Garner (66) found the dissolution time to be 8 to 15 minutes in thirteen instances, 15 to 60 minutes in eight tests, and from one to four hours with nine specimens. Morales-Otero and Pomales-Lebron (46) found that the time required for dissolution varied in tests with normal fibrin from 30 minutes to two and a half hours. Myers, Keefer, and Holmes reported that the average time for lysis with samples of blood from fourteen adults was one hour, the minimum time being 14 minutes and the maximum five hours. Waaler (78) stated that of tests made with specimens of blood from thirty-nine normal persons, thirty-four were classed as susceptible, and five as partially resistant. In a second article by Waaler (79) the blood of fifty of fifty-five normals were found to be susceptible and five partially resistant. Hadfield and associates stated that in tests with specimens of blood from twenty-eight adults none was totally resistant. Stuart-Harris (64) found the fibrin from 98.6 per cent of seventy-two persons to be susceptible. From his average results, and using a factor of standard deviation, he placed the limit of time for normal tests at 51 minutes.

From these results it may be seen that lysis of the fibrin clot of the blood of the great majority of normal individuals occurs in less than one hour, and commonly requires a considerably shorter time. Although each of the investigators has employed individ-

ual strains selected for the purpose but not standardized on the basis of any arbitrarily adopted unit of accurate measurement of fibrinolytic potency, the results are in general agreement.

On the basis of these findings, it may be estimated that the blood of approximately 85 to 90 per cent of normal healthy individuals may be arbitrarily classified as susceptible on the basis of tests in which the dissolution time is less than one hour.

In tests made with the blood of normal children, the data for age groups ranging from three to fifteen years of age are consonant with the findings in adults. Owing, however, to the frequency of upper respiratory infections in children during the winter months, it has been suggested that varying degrees of resistance may occur more frequently than in adults.

Among the acute diseases, directly referable to infection with hemolytic streptococci, immunological studies of the following conditions have been reported: Acute tonsillitis, with and without extension to mastoid, middle ear, or sinuses; scarlet fever, with and without complications; erysipelas; suppurative infections such as empyema, peritonitis, and abscesses in different locations; septicemia arising from different sources. The data to be given were obtained by consolidating all of the findings presented by various authors. Although the averages are not entirely in accord with the individual findings of each report, the differences are not sufficiently great to warrant a separate account of each.

In immunological studies it has been found that the development of antifibrinolytic properties may be demonstrable at variable times during the course of the disease up to as late as the third or fourth week in convalescence. The summarizing data which follow are in many instances derived from repeated examinations of the blood during acute illness and convalescence. However, in some of the cases, only one or two tests were made. The conclusions, therefore, are to some extent based on partially complete results which limit final conclusions.

*Acute tonsillitis.* Tillett, Edwards, and Garner (66), Myers, Keefer, and Holmes, Stuart-Harris (63, 64), and Tillett (68) have reported results obtained in forty-eight cases. In thirty-two of



the patients (67 per cent) an antifibrinolytic response was noted during convalescence. The time in the course of the disease at which the specific resistance developed varied from the first week to as late as the fifth week. In uncomplicated cases, the period of lag between the cessation of active disease and the detection of antifibrinolytic properties in the blood usually ranged from two to four weeks.

The degree of antifibrinolytic response was also found to vary in individual cases. The severity and extent of the infection were not infrequently found to be important factors not only in evoking the development of high antifibrinolytic response, but also in shortening the time of appearance of the specific immunity.

*Scarlet fever.* Tillett and associates, in eight cases, Dack and associates in forty-seven cases, Stuart-Harris, in fifty-eight cases, and Waaler in fifty-seven cases found the blood of 86 (50 per cent) out of 170 cases to possess antifibrinolytic properties, observed in most instances during convalescence. As in the patients with acute tonsillitis, the results in scarlet fever indicated that the development of antifibrinolysis becomes demonstrable usually within two to five weeks after the cessation of active disease. Dack and his associates noted that in the first test with some of the patients, the dissolution time was prolonged. Waaler found antifibrinolytic properties more frequently in cases complicated by otitis media and nephritis than in cases with adenitis or arthritis. Stuart-Harris obtained antifibrinolysis most frequently in cases complicated by arthritis, carditis, and nephritis.

In view of the fact that scarlet fever in recent years has been relatively mild, it seems reasonable to presume that the somewhat less frequent occurrence of antifibrinolytic immunity in patients with scarlet fever (50 per cent) than in those with acute tonsillitis (67 per cent) may be ascribed to differences in the severity of the infections. Cases of scarlet fever are usually hospitalized regardless of the degree of illness, whereas only relatively severe cases of acute follicular tonsillitis seek admission to hospitals, and become available for study. The reports,

referred to earlier, that scarlatinal strains of hemolytic streptococci possess less fibrinolytic potency than other strains, also suggests limitations in the antigenicity of the fibrinolysin.

*Erysipelas.* Combining the results obtained in different laboratories (66, 47, 63), resistance to fibrinolysis developed in 37 (80 per cent) of forty-six patients. Tillett, Edwards, and Garner noted that the development of resistance coincided in some cases with the cessation of the spread of the lesion. However, in other instances, the same authors observed a delay of one to three weeks in demonstrable antifibrinolysis. In ten of their cases, Myers, Keefer, and Holmes noted a high degree of resistance which was present during the period of active disease and persisted after recovery. In general, the antifibrinolytic response appeared in erysipelas at an earlier time during the course of the disease than in the uncomplicated cases of either acute tonsillitis or scarlet fever.

*Suppurative infections with and without septicemia.* This group includes cases of unusual severity. In some of the patients the occurrence of septicemia was reported. The mortality rate was high. In six fatal cases with septicemia, Tillett (68) found that none developed antifibrinolytic immunity. The patients died between the 6th and 25th day of disease. It is apparent that the survival period may not have been long enough to permit the appearance of the immune response. However, the limited data suggest that the occurrence of antifibrinolysis is less frequent in overwhelming infections than in local processes. Dack and his associates reported among the patients with scarlet fever, one fatal case in which the blood contained a high degree of antifibrinolytic resistance. Myers, Keefer, and Holmes also described a case which ended fatally with maximum resistance to lysis present in the blood. Of six cases with septicemia, which recovered, (4 reported by Tillett, and 2 by Stuart-Harris), three developed antifibrinolytic properties; the fibrin-clots of the other three remained susceptible even after the infection was overcome.

The findings with a miscellaneous group of infections, including cellulitis, empyema, mastoiditis, peritonsillar abscess, etc. may be collected from the several articles dealing with anti-

fibrinolytic immunity. Of twenty-two such cases, sixteen (73 per cent) developed the specific immune response.

A summary of the results just given is as follows:

	<i>Number of cases</i>	<i>Resistance present in per cent</i>
Acute tonsillitis.....	48	67
Scarlet fever.....	170	50
Erysipelas.....	46	80
Miscellaneous.....	22	73
Septicemia with recovery.....	6	50
Fatal cases.....	8	25
Normal individuals.....	165*	10-15

\* Approximate.

In a large number of the observations just summarized, the changes in the reaction of fibrin from susceptibility to resistance were demonstrated during the course of the diseases. The findings in serial tests with samples of blood from patients, who recovered, indicate that the fibrinolytic substance is frequently antigenic under the conditions of naturally occurring infections, and that the antifibrinolytic response is a specific immune reaction. However, additional observations suggest that insusceptibility to lysis may occur under conditions which are not referable to specific antibody response. The interpretation of single tests, carried out with plasma obtained during phases of active disease and convalescence will be subsequently discussed.

Furcolow and Fousek (14) performed eighty-four tests on seventy patients. In twenty-two instances no antifibrinolytic resistance was present. None of the latter had proven hemolytic streptococcus disease, although six were suspected. In twenty-five tests, the dissolution time ranged from one to three hours, which was interpreted as a suggestive but doubtful indication of resistance. Twenty-two of the patients either had proven hemolytic streptococcus infections or had been contacts. Antifibrinolytic resistance was marked in thirty-seven tests. Thirty-six of the cases had proven hemolytic streptococcus infections.

*Rheumatic fever.* Hadfield, Magee, and Perry made tests with the blood of forty-four children with rheumatic disease. The patients were divided into a group of twenty-one who had had recent active disease, and a second group of twenty-three quiescent cases. The first group was further subdivided into nine cases with sedimentation rate (red blood cells) above 20. Among them, five exhibited either maximum or partial resistance. Of twelve cases having had recent attacks but with a sedimentation rate below 20, five had maximum or partial resistance. The blood from each of the quiescent cases was susceptible to lysis.

Myers, Keefer, and Holmes made observations on thirty-four cases of rheumatic fever, twenty-nine of which either gave a history of a recent attack of acute respiratory infection or carried hemolytic streptococci in their throats at the time of admission to the hospital. Of these twenty-nine cases, the blood in twenty-seven possessed maximal antifibrinolytic resistance. Of the five remaining patients who had active disease but who gave no evidence either by history or by throat culture of having had hemolytic streptococcus infection, four possessed either maximal or partial resistance. The average time required for lysis in all the tests was nineteen hours.

Stuart-Harris (64) among twenty-two convalescent cases, found partial resistance in seven (32 per cent), and in tests with the blood of forty-eight active cases, twenty-nine (60 per cent) partial or complete resistance was present.

Waalder (79) tested the blood of seven patients with rheumatic fever, all of whom had acute infections of the throat. Six possessed maximal or partial resistance. He stated that when manifestations of active disease subsided, the antifibrinolytic property of the blood decreased.

Tillett (68) examined the blood of eight patients with active rheumatic fever, all of whom had had preceding acute upper respiratory infections. Six possessed maximum resistance, and two partial resistance.

Lippard and Johnson made observations on the blood of five cases (8 to 15 years of age). The dissolution time varied from 3 hours to maximum resistance. The authors also found high

titre of streptolysin antibodies in the same specimens of blood. However, the parallelism of antistreptolysin and antifibrinolysin was not quantitative, since the specimens with the highest titre of antistreptolysin did not exhibit the greatest degree of antifibrinolytic resistance. Stuart-Harris (63) also brought out the fact that titre of antistreptolysin and antifibrinolysin were not concomitantly present to the same degree.

*Summary of antifibrinolytic tests in patients with rheumatic fever*

	Number of cases	Resistance present in per cent
Active disease . . . . .	123	72
Quiescent disease . . . . .	45	15

From these results it is interesting to note that the findings obtained with cases of active rheumatic fever demonstrate that the frequency of the development of antifibrinolytic resistance (72 per cent) is essentially the same as that obtained in cases of acute tonsillitis without the visceral manifestations of rheumatic disease (67 per cent). Whether or not the rheumatic process has in itself the capacity to evoke an antifibrinolytic response or or whether upper respiratory tract infections due to hemolytic streptococci occurring frequently in rheumatic subjects elicit resistance to fibrin dissolution cannot be assayed from the results so far available. It is not within the scope of this article to discuss the broader subject of the possible relationship of hemolytic streptococci to rheumatic fever. However, the observations in cases of acute upper respiratory diseases of hemolytic streptococcal origin and also in cases of active rheumatic fever appear to be sufficiently consonant to indicate that the frequency with which antifibrinolytic properties develop in these disorders is comparable.

*Rheumatoid arthritis.* Myers, Keefer, and Holmes tested the blood from eleven cases; two had maximal resistance, and another, following acute sinusitis developed maximal resistance. The average dissolution time for the group was six hours, which is somewhat higher than the average of one hour for the normal controls, but considerably less than the average of nineteen hours for the cases of rheumatic fever.

Stuart-Harris in sixty cases of rheumatoid arthritis found resistance in six. Among ten cases of other types of chronic arthritis, no resistance was noted.

Waalder observed nineteen cases and recorded two as having 3+ resistance, four with 2+ resistance, four graded as 1+; nine were susceptible. He considered many of the reactions to be weak but suggested that resistance might have been more frequently encountered if the tests had been performed with samples of blood obtained earlier in the course of the disease. Neither the history of respiratory infections nor the results of bacteriological studies were reported.

*Gonococcal arthritis.* Of six cases studied by Myers and associates two had maximum resistance. The average dissolution time was five hours which is approximately the same as that obtained in cases of rheumatoid arthritis. Tillett and associates found in one case that normal susceptibility remained unchanged in tests repeatedly performed during sixty days of activity and convalescence. Stuart-Harris also noted susceptibility in one case of gonococcal arthritis.

*Still's disease.* In five children, Waalder observed no antifibrinolytic resistance.

*Summary of antifibrinolytic tests in patients with arthritis*

	Number of cases	Resistance present in per cent
Rheumatoid arthritis.....	90	14
Gonococcal arthritis.....	8	25
Still's disease.....	5	0

The findings with the arthritic group are significant when contrasted with the results obtained in rheumatic fever. It is also interesting to note that the frequency of antifibrinolytic resistance was slightly greater than in normal individuals. A discussion of the possible significance of these results will be reserved until the findings in other diseases are described. However, from the standpoint of critical analysis, it would appear to be necessary to exclude the possibility of a relatively recent hemolytic streptococcus infection—whether causal or incidental—as the incitant of the antifibrinolytic response in order to

interpret the findings obtained in chronic disorders of uncertain etiology.

*Acute Nephritis.* In five patients, all of whom had previously suffered from acute tonsillitis due to hemolytic streptococci, four developed moderate to maximal resistance (68). Waaler (78) commented upon the frequency of antifibrinolysis in seven cases. In four cases, Myers and associates found the average dissolution time to be six hours, but did not comment upon the occurrence of antecedent respiratory infection. Similarly, Stuart-Harris found the fibrin-clot in two cases to be susceptible. No bacteriological details were given.

*Recurrent tropical lymphangitis.* Morales-Otero and Pomales-Lebron (46) in a study of the relationship of hemolytic streptococci to tropical lymphangitis tested for the presence of antifibrinolytic properties in the blood of fourteen patients suffering from this disease. They found maximum antifibrinolytic resistance in 7 instances, moderate resistance in 3. In the remaining 4 cases resistance was either absent or doubtful. They reported that the resistance was usually most marked early in the disease, gradually decreased during convalescence, and rapidly reappeared with a recurring attack of lymphangitis.

*Bacterial endocarditis.* Myers and associates studied two cases due to *Streptococcus viridans* from each of which the fibrin-clot exhibited maximum resistance. A third case, with infection due to an indifferent streptococcus, was found in repeated examinations to be without antifibrinolytic properties.

Waaler (79) tested the blood of four cases which were due to *Streptococcus viridans*. Three of the four possessed antifibrinolytic properties. A fifth case, due to a fecal streptococcus, gave tests rated as 2+ resistance.

Stuart-Harris reported observations in two cases which were due to *Streptococcus viridans*. One of the patients, who had previously suffered from rheumatic fever, developed partial resistance. The fibrin-clot of the other was susceptible; and at autopsy no signs of rheumatic fever were noted. In a third case of undetermined bacterial etiology, no antifibrinolytic resistance was present.

The high incidence of antifibrinolytic properties in the blood

of patients with endocarditis due to *Streptococcus viridans* is an interesting finding, the interpretation of which is not apparent. If the resistance to lysis is dependent upon the presence of specific immune properties, the antifibrinolytic response appears to be evoked either by green streptococci or in association with the underlying rheumatic disease. The possible influence of non-specific factors in antifibrinolysis will be presently considered. It is interesting to note in passing that both McEwen and Coburn have reported in personal communications that the antistreptolysin titre of the serum of patients with bacterial endocarditis is usually not increased.

*Diseases not associated with hemolytic streptococci.*—It is unnecessary to consider individually the large number of diseases which have been used for comparison with infections due to hemolytic streptococci. The control groups have consisted of diseases of diverse bacterial etiology, such as pneumonia, tuberculosis, typhoid fever, diphtheria, staphylococcal infections, etc. The findings in pneumonia will be considered separately. With respect to the other non-streptococcal diseases, the results have not indicated that any specific type of disorder is characterized by the presence of antifibrinolytic properties in the blood. However, it is of interest to note that the average degree of antifibrinolysis in the control group of patients is somewhat greater than that found in normal persons. For example, Myers, Keefer, and Holmes reported the average dissolution time of the two groups to be four and one-half hours and one hour, respectively. Stuart-Harris inquired into the past history of the non-streptococcal cases which possessed antifibrinolytic properties, and in several instances noted that a preceding attack of tonsillitis or rheumatic fever may have accounted for the resistance to lysis. However, even though occurrence of a concomitant or preceding hemolytic streptococcus infection may be responsible, in some instances, for the antifibrinolytic response of patients with non-streptococcal diseases, there is suggestive evidence that alteration in the blood associated with the acute active phase of infection may inactivate the fibrinolytic process. In this connection the findings in pneumonia are of interest.

*Pneumonia.* Waaler (78) reported six cases of pneumonia,



in which the fibrin-clot was resistant. In one of the cases the resistance persisted for two months. Of five cases of pneumococcus pneumonia in adults reported by Tillett, Edwards, and Garner, the fibrin-clot of four was found to be susceptible both during the phase of acute, active disease and also during several weeks of convalescence. In one patient, however, the blood obtained during active pneumonia exhibited maximum resistance, but within a few days after recovery there was a sudden and complete loss of resistance. The rapid disappearance of the antifibrinolytic properties in this patient, associated with critical recovery, contrasted markedly with the gradual reduction over weeks or months of the resistance in patients with proven hemolytic streptococcus infections. Stuart-Harris studied four cases of pneumococcus infection, two of which were pneumonia, one of mastoiditis, and one of pericarditis. The fibrin-clot, in each instance, was found to be susceptible. The tests apparently were performed during acute illness although no specific statements are made as to the time in the course of the illness at which the specimens of blood were obtained. Myers, Keefer, and Holmes included cases of pneumonia in their large group of non-streptococcal diseases. As previously mentioned, the average dissolution time of the whole control group was four and one-half hours. The results with the blood from patients with pneumonia were not separated from the others.

The conflicting results with pneumonia consist of the uniform finding by Waaler of high antifibrinolytic resistance, and the negative results of others, with the exception of the one case mentioned. Waaler concluded from his studies of patients with bacterial endocarditis and pneumonia that the occurrence of antifibrinolytic properties in the blood of patients was not decisive evidence of hemolytic streptococcus infections.

Interesting information is obtained from the studies of Lippard and Johnson concerning children with pneumonia. They noted that, in the youngest patients, maximum resistance was present early in the disease but abruptly disappeared three to thirteen days after onset. This finding was, however, not regularly obtained in all of the children with bronchopneumonia. Boisvert

reported that in the pediatric age group, the majority of patients with pneumococcus pneumonia possessed antifibrinolytic resistance during the period of active disease but rapidly lost it after recovery.

An interpretation of the data obtained in pneumonia is not apparent at the present time. The factor of age of the patient may be important. In addition, certain other possibilities warrant consideration. The results obtained by some of the investigators were characterized by the fact that insusceptibility to fibrinolysis did not gradually appear over periods of time after the beginning of the infection, as occurs in usual immunological responses. On the contrary, the high antifibrinolytic potency of early tests was followed by abrupt loss instead of gradual disappearance. In view of this particular course of events, the possibility suggests itself that the inactivating effect exerted on the fibrinolysin of hemolytic streptococci by the blood of some cases of pneumonia is not dependent upon immunologically specific antibody but to non-specific substances present in the blood during acute illness and rapidly lost during recovery.<sup>1</sup> On the basis that the fibrinolysin is an enzyme, it is interesting to speculate whether antienzymic effects comparable to the rise of antitrypsin which occurs during acute infection might account for the inactivation of the fibrinolytic enzyme. An additional example of the effect of blood from cases of acute illness on hemolytic streptococci is furnished by the report of Tillett (70) who found that the serum of patients with pneumonia and other types of infection is highly streptococcal, but the property is rapidly lost following cessation of active disease.

On the basis of the present information, the interpretation of single tests may be summarized as follows:

During active acute infections of streptococcal or non-streptococcal origin. *In children*, antifibrinolytic properties are fre-

<sup>1</sup> In a recent personal communication Dr. P. L. Boisvert of the Department of Pediatrics of Yale University School of Medicine outlined studies of antifibrinolytic immunity which are in progress. It would be premature to comment in this article on his extensive but uncompleted data. However, the findings, up to the present time, differentiate, in the pediatric age groups, between the specific immunity and probable non-specific inactivation.

quently present (Lippard and Johnson; Boisvert). *In adults*, antifibrinolytic properties are frequently absent (66, 47, 63) but have been noted in pneumococcus pneumonia (78).

During convalescence, the development of antifibrinolytic properties, following non-streptococcal disease, has not been reported; following infections due to hemolytic streptococci, antifibrinolytic properties have appeared in approximately 60 to 80 per cent of the cases.

The fact that the dissolution time in 10 to 15 per cent of normal persons is prolonged may account for the findings in which "moderate resistance" remains unchanged during acute illness and recovery. Since "maximum resistance" has not been noted in normal healthy persons, its occurrence during convalescence is strong presumptive evidence of relatively recent infection due to a hemolytic streptococcus.

Tillett and Garner reported that the serum from convalescent patients, the fibrin-clot of whose blood was resistant to dissolution, conferred antifibrinolytic properties when added to normal plasma. Demonstration of the presence of antifibrinolysin in the serum suggested that specific resistance to fibrinolysis was not dependent upon properties of the fibrin substrate itself. Van Deventer (75) isolated fibrinogen from the blood of several individuals. Tests with the plasma-clot of these subjects indicated varying degrees of resistance. However, when fibrinogen-thrombin preparations were used, no differences in susceptibility were noted. The same author (75a, 76) tested twenty-eight commercial antistreptococcus sera by "passive transfer" to normal human fibrin. He added the fibrinolysin in arbitrarily designated units to dilutions of sera and incubated the mixtures for 3 hours before adding to the fibrin constituents of the test. Six of the sera were found to possess high titres of antifibrinolytic antibodies. He added potent antisera to rabbit and monkey blood, allowed them to clot, and was able to demonstrate the antifibrinolysin in the serum expressed from the clots. Van Deventer also attempted to immunize rabbits with fibrinolysin, using several cultural preparations as antigens. However, the sera of the animals, even after many injections, failed to exhibit

antifibrinolytic properties when tested with susceptible human fibrin. Schmidt (57) titrated samples of antistreptococcus horse sera, and according to the quantitative procedures, which he described, 0.0025 cc. of highly potent sera were capable of inhibiting fibrinolysis.

In some respects the use of serum in testing for antifibrinolytic resistance is more advantageous for quantitative titration than is plasma. However, factors which have not up to the present time been studied in detail may condition the serological results. Because of an insufficiency of experimental data it is unnecessary to discuss the problem in detail. However, mention may be made of the fact that the thrombin contained in sera may be sufficiently high to coagulate, either wholly or partially, the substrate without the addition of  $\text{CaCl}_2$  to oxalated plasma, or of specially prepared thrombin to fibrinogen. Since both thrombin and antibodies are closely associated with the globulin fraction of blood, the possibility suggests itself that the thrombin of immune sera might carry antifibrinolysin into the forming fibrin, whereas the thrombin of normal sources results in the formation of susceptible fibrin. It is apparent that the standardization of serological procedures must await additional studies.

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# COLIFORM BACTERIA

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The oldest members of the coliform group of bacteria, as the reviewer conceives it, are *Klebsiella pneumoniae*, or Friedländer's bacillus, described in 1882, and *K. rhinoscleromatis*, which v.Fritsch recorded the same year. Next come *Escherichia coli* and *Aerobacter aerogenes*, both of which were ushered into the bacteriological world in 1885 by Escherich. Somewhat younger is *A. cloacae*, described in 1890 by Jordan. *Proteus morgani*, the problem child of the group, dates from 1908 and the juveniles are *E. freundii*, recorded in Braak's Delft thesis of 1928, and *K. paralytica*, the etiological agent of "moose disease," described in 1932 by Cahn, Wallace, and Thomas.

These are the principal members of the coliform group as listed in the fifth edition of Bergey's Manual of Determinative Bacteriology (12), a great simplification of the genera *Escherichia*, *Aerobacter* and *Klebsiella* given in the fourth edition (1934)

which contained 35 species. In the latest edition these three genera comprise but ten species.<sup>1</sup>

With this simplification we are in heartiest accord, but the problem has several aspects. Among them, the principle of simplification, or "lumping," as Skinner and Brudnoy (149) term it, must be defended. The inclusion of the genus *Klebsiella*<sup>2</sup> with the coliform bacteria has to be explained. To claim the Morgan bacillus as a coliform organism will require justification. The broadening of the concept of *Escherichia coli* to include such forms as *E. coli-mutabile* and the paracolon bacilli which ferment lactose slowly or not at all is a new development and there are many who will want to know how the "coli-aerogenes intermediates" (180, 165, 25, 123) are classified and why.

#### THE TERM "COLIFORM"

The term "coliform" has long been in use by British bacteriologists (4, 23, 59, 92, 100, 132, 133, 160). In America, Breed and Norton (16) suggested the term to describe the lactose-fermenting bacteria used as a measure of the pollution of water. In 1937 H. E. Jordan advised that as Editor of the Journal of the American Water Works Association his policy would be to substitute "coliform" bacteria for "*B. coli*" or "colon group" in papers submitted to him (70). As Jordan also stated, the term "*coli-aerogenes*" continues as official in water analysis since the eighth edition of Standard Methods of Water Analysis (157) uses it. And "*Escherichia-Aerobacter*" is also official since that is the terminology employed in the sixth edition of Standard Methods of Milk Analysis (156).

#### SEPARATION OF FRIEDLÄNDER GROUP FROM OTHER COLIFORM BACTERIA

As noted above, the first members of the coliform group to be described were *Klebsiella pneumoniae*, from acute fibrinous

<sup>1</sup> The other three species are *Klebsiella ozaenae*, described by Abel in 1893; *K. granulomatis* of Aragás and Vianna (1912); and *K. capsulata* recorded in 1889 by Pfeiffer.

<sup>2</sup> These organisms have also often been called the "*Bacillus mucosus capsulatus* group."

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pneumonia, and *K. rhinoscleromatis*, from rhinoscleroma. The typhoid bacillus had been described less than two years earlier and the emphasis in early bacteriology on its medical aspects favored the finding of pathogens. But even at this time the inclusion of the encapsulated forms found in the upper respiratory tract in a group with coliform bacteria from the intestine and from milk was urged (31). From the first all studies of the gram-negative, aerobic, encapsulated bacilli, of which the Friedländer bacillus is the type known to pathologists, have also included *Aerobacter aerogenes*<sup>3</sup> and all studies of coliform bacteria have likewise included *A. aerogenes*.

The close relationship of *Klebsiella* and *Aerobacter* has been asserted or demonstrated down to the present time (72, 74, 75, 76, 77, 41, 59, 170, 93). Perkins maintained that the prototype of the encapsulated group was *A. aerogenes* and that the other members were variants which had lost the power, in whole or in part through modification in environment (125), to ferment certain sugars. Edwards stated that *A. aerogenes* is so closely related to the other encapsulated forms that they should be classified in the same genus (41).

Notwithstanding, the Friedländer group and the rest of the coliform bacteria have been kept apart in the minds of most bacteriologists. This unfortunate point of view has separated two groups which must be considered together and it has minimized our understanding of the potentialities for pathogenicity possessed by *Escherichia* and *Aerobacter*. As we shall see, these forms, particularly *Escherichia*, are important in pathology, especially in infections of the urinary tract, in all age groups, and of the gastro-intestinal tract in the very young.

As a result we shall have to trace the steps in the history of the classification of coliform bacteria bearing in mind that *Klebsiella* is probably not under consideration and that the emphasis is mainly on the sanitary aspects of the organisms in question. Later we shall bring the Friedländer group back into the dis-

<sup>3</sup> In our own discussion in this review the terminology of the Bergey Manual will be employed but we believe that the use of one genus for all coliform bacteria is biologically more sound than the use of three.

cussion for it has a definite place in the concept of the coliform group.

#### DEVELOPMENT OF THE CLASSIFICATION AND CHARACTERIZATION OF COLIFORM BACTERIA

Escherich (42, 43) characterized *Bacterium coli-commune* as a bacillus of feeble motility which coagulated milk but did not liquefy gelatin; which fermented milk-sugar and grape-sugar with the disengagement of gas; which produced a moist growth on potato of a color varying from corn yellow to pea yellow; and which produced in animals a rapidly fatal disease characterized by diarrhea, somnolence and coma. *Aerobacter aerogenes* was first described as *Bacterium lactis-aerogenes* by Escherich who noted that it was shorter and plumper than *B. coli*, coagulated milk more actively and was non-motile. He stated that it fermented milk-sugar, cane-sugar and grape-sugar, both aerobically and anaerobically, and he pointed out the prominence of lactic acid among the products of fermentation.

A decade later Theobald Smith (150) suggested a biological division of *E. coli* when he redefined *B. coli-communis*<sup>4</sup> stating it could be divided into alpha and beta varieties based on sucrose fermentation. Smith is usually credited with pioneer work in determining the gas ratios produced in the fermentations. The results were corrected by later workers using more delicate methods; but it is curious that this promising field has not been exploited more thoroughly. Durham (39) named the sucrose-fermenting variety of the colon bacillus *Bacillus coli-communior*. Among the other species of coliform bacteria early described and frequently encountered in the literature may be mentioned the "Milchsäurebacterium" of Hueppe (1884) described in 1885 by Zopf as *Bacterium acidi-lactici*, and Flügge's *B. neapolitanum* (1885). These two species and *B. communior* appear in Bergey (12) as varieties of *Escherichia coli*.

MacConkey (96, 97) first placed classification of coliform organisms on a comprehensive, biochemical basis. He established

<sup>4</sup> In the review of a reference its terminology is used even if outdated or incorrect as some are.

four primary groups of the lactose fermenters based on the fermentation of sucrose and dulcitol. These groups were further subdivided on the basis of tests for motility, indole production, gelatin liquefaction, the Voges-Proskauer reaction and the dissimilation of adonitol, inositol and inulin. MacConkey's scheme called for 128 types of which he actually isolated 36. By 1928 other workers had described 35 additional types, making a total of 71 to be recognized by that date (133). Climaxing taxonomic work of the MacConkey type, Mackie (100) studied the coliform bacteria of feces and urine, forming four principal groups on the basis of gas production, indole production and inositol fermentation. Subdivisions of these groups allowed for many types of which 73 were found. This contribution is of value today because of its emphasis on the use of inositol and as a further illustration of the difficulties encountered by taxonomists whose schemes of classification proceed on the 2-4-8-16-32 ... or "2<sup>n</sup> principle."

As pointed out by Topley and Wilson (170), one important correlation between biochemical activity and natural habitat was early recognized. The *A. aerogenes* type was found to be a relatively infrequent inhabitant of the intestine, but was frequently isolated from certain grasses and from the soil, while *B. coli-communior* and *B. coli-communis* were noted to be typically intestinal parasites (185). This correlation was of practical as well as of theoretical importance. The presence or absence of "*B. coli*" in water supplies, and the relative number of this organism if present, soon came to be recognized as a very valuable indication of the presence and degree of fecal pollution, and it became desirable to differentiate between those types which were of intestinal origin, and those which might occur in unpolluted waters.

Thus, editions of Standard Methods of Water Analysis published in the second decade of this century included a classification in which "the following group reactions indicate the source of the culture with a high degree of probability:" and there followed a classification of "fecal" *B. coli*, "fecal" *B. aerogenes*, *B. aerogenes* probably not fecal, and *B. cloacae* which might or might not be

fecal, based on the methyl-red and Voges-Proskauer reactions, the liquefaction of gelatin, the production of indole and the fermentation of sucrose and adonitol. This material has not appeared in recent editions because no confirmation could be found for the early reports that *A. aerogenes* of intestinal origin fermented adonitol whereas *A. aerogenes* from grains and soil did not. Indeed, as we shall see, the prolonged search for tests which differentiate between fecal and non-fecal coliform bacteria has so far been in vain.

As early as 1912 principles of simplification of coliform classification were beginning to appear. Thus Howe (64) showed that there was no correlation between certain features, such as motility, fermentation of dulcitol and mannitol, indole production and nitrate reduction; and he recognized only two species, *B. communis* and *B. communior*. Kligler (79) subdivided the lactose fermenters according to their sucrose and salicin reactions rather than by sucrose and dulcitol reactions, as proposed by MacConkey, since sucrose-salicin sub-groups correlated more highly with the indole, Voges-Proskauer and gelatin reactions than did the sucrose-dulcitol subgroups. The chief emphasis in coliform taxonomy on simplification through the application of statistical methods of correlation of related characters has been expressed by Levine (89, 90). His classification also was one of the first to make use of the Voges-Proskauer test as the primary feature of division of the strains studied.

From quite another point of view Clemesha (28) grouped coliform bacteria according to the resistance shown toward storage. This work represents the most important early contribution to the ecology of the coliform bacteria, possibly of any group, and treats in detail such problems as viability, competition and succession.

Although the first edition of Bergey's Manual of Determinative Bacteriology (1923) allocated 22 species to the genus *Escherichia*, six to *Aerobacter*, and six to *Encapsulatus* (*Klebsiella*) classified mainly on the basis of reactions with fermentable substances, ground had been broken for a somewhat different and more basic approach to taxonomy. The Committee of the Society of Amer-

ican Bacteriologists on the Characterization and Classification of Bacterial Types, appointed at the 1915 annual meeting at Urbana, projected, among other tasks, a study of the colony-typhoid group of bacteria, "a group which, together with certain sharply defined species, includes many puzzling intermediate forms, difficult of classification and yet of fundamental medical and sanitary importance" (186). Out of the interest of this committee, two sets of data emerged dealing with coliform bacteria.

These have been summarized by Yale<sup>5</sup> as follows: "During the period 1914 to 1921, Rogers and associates published a series of outstanding papers on the characteristics and distribution of the coliform group. An especially important contribution was the separation of the group into a low ratio section in which the  $\text{CO}_2\text{:H}_2$  ratio was approximately 1:1 and a high ratio group in which it was approximately 2:1. In a report summarizing these studies in 1921, Rogers made this statement, 'So far as data are available, the low ratio or *B. coli* group appears to be a very definite and circumscribed entity and there is no apparent reason for separating it into species.' In addition he recognized *B. aerogenes* and *B. cloacae* as separate species in the high ratio group."

"Winslow, Kligler and Rothberg (186) made extensive classification studies and decided that in the low ratio group, only four species were justified (*Bacterium coli*, *B. communior*, *B. acidilactici* and *B. neapolitanus*). In the high ratio group, two species were accepted (*Bacterium aerogenes* and *B. cloacae*)."

The methyl-red test (27) was a logical outcome of work in Rogers' laboratory on the fundamental nature of the fermentation of glucose by coliform bacteria. These Washington workers found that the low gas-ratio *E. coli* section produces under suitable conditions a lower pH in glucose broth than do the high gas-ratio *A. aerogenes* organisms. As a result, the former give a red color to methyl red (+) and the latter a yellow color (-)

<sup>5</sup> Round Table Discussion on the coliform group of bacteria, 39th annual meeting, Society of American Bacteriologists, Washington, D. C., December 28, 1938.

in the glucose broth. The formation of acetyl-methyl-carbinol, demonstrable by the Voges-Proskauer test, occurs when *A. aerogenes* is grown in glucose broth but does not occur with *E. coli*. This inverse correlation (M.R. + V.P. - and M.R. - V.P. +) was emphasized by Levine (88). For some years it was held to be well-nigh perfect, but it is now known that coliform organisms occur which are positive to both tests and also those which are negative to both. Thus Stuart and co-workers (161) found that almost ten per cent of their coliform cultures did not correlate.

Another important development has been the recognition of "intermediates," organisms resembling both *E. coli* and *A. aerogenes* but identical with neither. In a study of the utilization of simple nitrogenous compounds, Koser (82) observed that *A. aerogenes* was able to multiply and grow luxuriantly in a medium containing uric acid as the only source of nitrogen. In this medium *E. coli* failed to develop. Later work by Koser (83) developed another, and better, utilization test, i.e., growth in a medium in which the only available carbon source is the citrate radical.\* In this medium *A. aerogenes* develops very well, whereas *E. coli* fails to grow noticeably (140). It was soon found that some methyl-red-positive organisms, presumably *E. coli*, grew as well in the citrate medium as *A. aerogenes* (85). For a time such organisms were spoken of as "soil coli" and there was again revived the hope that sanitarians had a test which would set off fecal from non-fecal organisms. Such organisms are now known as "intermediates" and are found in feces as well as in man's environment.

Werkman and Gillen proposed the genus *Citrobacter* for coliform bacteria producing trimethylene glycol, and described seven species (180). Since such bacteria utilize citrate and are methyl-red-positive, it was thought that "*coli-aerogenes* intermediates" might be allocated to the genus *Citrobacter*, but the genus has not

\* Pesch (1921) evidently somewhat anticipated the work of Koser on citrate utilization but apparently did not follow it up with further investigations, and he dealt more primarily with other members of the colon-typhoid group than the coliform bacteria.

been recognized by the leading taxonomists. Tittsler and Sandholzer (165), and Carpenter and Fulton (25), favor classifying "intermediates" with *Escherichia* and *Aerobacter*; Minkewitsch (106) calls them *B. coli-citrovorum* Koser; and Parr (123) suggests that they be made a species in the genus *Bacterium*, which would include also *B. coli*, *B. aerogenes* and *B. cloacae*. The fifth edition of Bergey's Manual designates them as *Escherichia freundii*.

In an analysis of data presented in recent papers (1924-1937) dealing with coliform bacteria, Parr (123) found that the reactions earlier used for classification, such as the fermentation of sucrose, dulcitol, salicin, and raffinose, have largely been replaced by other tests. If this analysis be brought down to date it will be found that in 22 of the most recent projects the following tests have been used to establish coliform classification:

	<i>times</i>
Indole production . . . . .	15
Methyl-red reaction . . . . .	20
Voges-Proskauer reaction . . . . .	22
Citrate utilization . . . . .	20
Uric acid utilization . . . . .	6
Cellobiose fermentation . . . . .	4
Gelatin liquefaction . . . . .	3
Eijkman test . . . . .	2
H <sub>2</sub> S production . . . . .	2
Sucrose fermentation . . . . .	1
Inositol fermentation . . . . .	1
Alpha-methyl-d-glucoside fermentation . . . . .	1

There is, therefore, justification for the creation of the "Imvic" quartet of tests (119). Imvic is a mnemonic which fixes in order the four tests now in greatest use in classifying coliform bacteria: (I) indole production; (M) methyl-red reaction; (V) Voges-Proskauer test; and (C) the utilization of citrate as a sole carbon source. Four characters give 16 possible combinations. Three of these (+ + - -, - + - -, + - - -) Parr called the *E. coli* section, three (- - + +, - - + -, - - - +) the *A. aerogenes* section, and ten (+ + + -, + + - +, - + - +, + - + -, - - - -, + - - +, - + + -, + + + +,



+ - + +, - + + +) constitute the "intermediate" section, and he stated that 14 of the 16 types have been reported.

Recently, Stuart and co-workers (161) used the Imvic group of tests plus the fermentation of cellobiose in the study of a large series of coliform bacteria. Cellobiose may be fermented with acid production or with the production of both acid and gas, or it may not be fermented at all. Hence, recording three possibilities for cellobiose brings the possibilities of the Stuart scheme to 48 types of which the Brown University group found 21 among the 3247 cultures studied. These could be assigned to nine of the 16 Imvic types. Of the 48 Stuart types Parr has found 16, Skinner and Brudnoy (149) 14, and Oeser (115) 13.

Malcolm (103) classified 1636 coliform strains isolated from milk and bovine feces into eight groups on the basis of the Voges-Proskauer test, citrate utilization, inositol fermentation and indole production. He called one group *B. coli*, three groups "intermediates," one *B. friedländeri*, one *B. cloacae*, one *B. oxytocus*, and one *B. aerogenes*, and he insisted that all should belong to one genus. In addition he encountered 39 anomalous strains which did not fit into his scheme of classification. This work illustrates again the difficulty of classification in this field. The group is so complex and intergrading that each form recovered cannot be assigned an exact name without making the number of such names well-nigh legion. It is refreshing, therefore, to find that Yale in the fifth edition of Bergey's Manual has kept the number of species small, listing some forms as varieties and disregarding scores of names that have confused workers down to the present day.

That the field of biochemical classification has been by no means exhausted is shown by Mitchell and Levine (109), who studied coliform bacteria to determine if nitrogen utilization was as distinctive for differentiation as carbohydrate dissimilation is thought to be. Nucleic acid and its degradation products were employed as nitrogen sources in synthetic media with glucose as the carbohydrate source and indicator. Over 350 strains were tested with yeast nucleic acid, uric acid, allantoin, hydantoin, uracil, urea, adenine and xanthine. Organisms giving positive

reactions with yeast nucleic acid, uric acid, allantoin and hydan-toin correlated with the positive Voges-Proskauer test and formed the section *Aerobacter*. *Escherichia* and the "intermediates" were negative to these tests<sup>7</sup> but among them *Escherichia* utilized uracil and failed to utilize urea, whereas the "intermediates" metabolized urea but could not break down uracil. Since both *Escherichia* and *Aerogenes* can utilize uracil and the "intermediates" cannot, Mitchell and Levine feel that they have additional evidence that the "intermediates" should constitute a separate genus. Other evidence is that many "intermediates" produce hydrogen sulphide and those tested yield trimethylene glycol in a suitable medium.

Returning to the Friedländer group it is to be noted that these encapsulated coliform bacteria have not been satisfactorily classified on any such basis as has been used for the other members of the group. Perkins (125) stated that organisms of this group which show no fermentative power are probably degenerate rather than definite entities and can in many cases be reactivated to their original type. Fitzgerald (46) studied 44 cultures of *Bacillus mucosus-capsulatus* and found satisfactory biochemical classification difficult. He believed that mutations, based on the necessity of maintaining a parasitic existence, have caused gram-negative bacilli, found normally in the body elsewhere than in the intestinal tract, to develop capsules for protection, and a new group has arisen designated as *B. capsulatus-mucosus*, connected by the varieties *B. aerogenes* and *B. acidilactici* with the non-encapsulated gram-negative bacilli belonging to the colon group.

Winslow, Kligler and Rothberg (186) in commenting on the encapsulated pathogenic forms, said: "It seems evident, either that we are dealing with an extraordinarily variable group, or that forms which are not really related have been identified as of this type merely because of possession of capsule." It should be recalled that *Escherichia coli* not infrequently occurs heavily encapsulated (151, 118).

<sup>7</sup> Parr (123) maintains that some "intermediates" are Voges-Proskauer positive.

THE TRIBE ESCHERICHEAE AND RELATED FORMS ACCORDING TO  
BERGEY

In the fifth edition of Bergey's Manual of Determinative Bacteriology (12)<sup>s</sup> Family X. *Enterobacteriaceae* Rahn is divided into five tribes, viz.,

Tribe I. *Eschericheae*: 3 genera, 10 species.

Tribe II. *Erwineae*: 1 genus, 13 species.

Tribe III. *Serrateae*: 1 genus, 6 species.

Tribe IV. *Proteae*: 1 genus, 8 species.

Tribe V. *Salmonelleae*: 3 genera, 65 species.

The three genera of the tribe *Salmonelleae* are *Salmonella* with 37 species and 12 additional varieties, *Eberthella* with 14 species, and *Shigella* with 14 species. With the exception of *Proteus morgani*, the coliform group is found in and includes all of the tribe *Eschericheae* which we shall next consider.

*Eschericheae* trib. nov.—ferment dextrose and lactose with the formation of acid and visible gas. In only one genus, *Aerobacter*, is gelatin liquefied and that but slowly.

"Genus I. *Escherichia*.—Methyl red test positive. Voges-Proskauer test negative. Citric acid may or may not be used as sole source of carbon.

I. Citric acid not utilized as sole source of carbon.

A. Hydrogen sulphide not produced.

1. *Escherichia coli*.

II. Citric acid utilized as sole source of carbon.

A. Hydrogen sulphide produced.

2. *Escherichia freundii*.

Genus II. *Aerobacter*.—Methyl red test negative. Voges-Proskauer test positive. Citric acid used as sole source of carbon.

I. Glycerol fermented with acid and gas.

A. Gelatin not liquefied.

1. *Aerobacter aerogenes*.

II. Glycerol not fermented with acid and gas.

A. Gelatin liquefied.

2. *Aerobacter cloacae*.

Genus III. *Klebsiella*.—Methyl red test usually positive. Voges-Proskauer test usually negative. Citric acid usually (?) used as sole

<sup>s</sup> For both galley and page proofs of the new Bergey Manual dealing with the family *Enterobacteriaceae* I am indebted to Dr. Robert S. Breed.

source of carbon. Capsulated forms from respiratory and other mucous membrane regions.

- I. Litmus milk acid, but not coagulated.
  - A. No acid and gas from maltose or mannitol.
    1. *Klebsiella pneumoniae*.
  - B. Acid and gas from maltose and mannitol.
    2. *Klebsiella ozaenae*.
- II. Litmus milk acid and coagulated.
  3. *Klebsiella granulomatis*.
  4. *Klebsiella capsulata*.
  5. *Klebsiella paralytica*.
- III. Litmus milk unchanged.
  6. *Klebsiella rhinoscleromatis*.<sup>9</sup>

#### OCURRENCE AND SIGNIFICANCE OF COLIFORM BACTERIA, INCLUDING PATHOLOGY

In the discussion which follows an effort will be made to present and preserve the ecological point of view which should help to explain why encapsulated coliform bacteria occur in one environment and non-encapsulated in another. A shift of environment may provide opportunity for the development in large numbers of species which survive in small numbers and with difficulty in another milieu. Or, if we are dealing with pure cultures, changes in environment may favor the survival of the descendants of one variant or mutant over those of another. Natural selection has probably not caused organic evolution but it is one of the most important factors in its direction. What happens to an association of bacteria or to a pure culture of a single organism with capacity for variation or mutation will in large part depend on the environment.

The entire family *Enterobacteriaceae* Rahn, with the exception of the plant parasites, and the red pigment-producing chromogens, are thought of as intestinal bacteria, having, however, in the case of the more saprophytic species a considerable distribution outside the body of man and animal in nature.

*Animal pathology.* Very little attention was paid to the coliform group in veterinary medicine until comparatively recently.

<sup>9</sup> From page proof, fifth edition, (12), *Manual of Determinative Bacteriology*.

Citations include *Proteus morgani* infections of zoo animals, gaseous emphysema, abortion in sheep, a disease of white rats associated with parasitic infestation, "moose disease," diarrhea in foals and in young pigeons, calf "scours," infectious enteritis of young lambs, a fatal disease of carp, metritis of mares, pneumonia in beavers, mastitis in cows, infectious diarrhea of chicks and abortion in ewes.

TenBroeck recently stated (1938) that it is extremely difficult to evaluate the importance of the colon group in animal pathology but that they take the place of the streptococci in man, i.e., they are often secondary invaders that complicate infection. Plastridge (1938) involved coliform organisms as responsible for navel ill in chicks, navel ill in calves, and a limited number of acute cases of mastitis in cows, and Hitchner (1938) stated that slow lactose-fermenting coliform bacteria have caused several epidemics of intestinal disease among chicks in Maine.

It will be noted that coliform bacteria produce "scours" in calves, diarrhea in foals and pigeons, infectious enteritis in young lambs, and infectious diarrhea in chicks. This pathogenicity for the young of animals is significant. Calf "scours" has been the subject of a classical investigation by the Rockefeller group at Princeton. The coliform bacteria concerned are usually in the mucoid phase (151, 95) but they may not be (Dollahite, 1938). Certain points are significant. The disease is produced by a soluble, diffusible substance of exotoxic nature (153, 131). This toxin is a capillary toxin and is far less effective in the usual laboratory animal than in the calf. Colostrum or maternal serum will protect the calf. The disease is one of intoxication and diarrhea with resultant dehydration. "If we put all the facts together the inference seems admissible that scours is associated with special races of *Bacillus coli* and that such races are developed and maintained in large herds. This will account for the different races of *Bacillus coli* which have been charged by other observers as the cause of scours. Each large herd through the presence of calves below par at birth may thus develop and maintain its own type of scours organism which, however, is not virulent enough to make any headway in naturally

strong calves properly cared for as regards food and housing'' (Smith and Orcutt, 151). Somewhat the same view is held by Lovell (131) who concluded that special races of *Bact. coli* pathogenic for young calves exist, but that more than one race may be present in a herd and sometimes more than one type may be isolated from an individual calf.

The importance of this disease may be inferred from the statement of Dollahite (1938), released by Schoening (1938), that during the first five months of 1938 in a large government-owned dairy 68 calves were born, of which 33 (49 per cent) died with acute dysentery before they were five days old. Many of them died within 48 hours after birth.

In this outbreak *Escherichia communior*, *E. acidi-lactici*, and rarely *E. coli*, were encountered with *E. communior* occurring in about 60 per cent of the cases. From June 15 to November 15, 1938, cow serum prepared with a pooled *E. communior*-*E. acidi-lactici* antigen was given intravenously twice on successive days to 35 new-born calves with the result that none of the calves died, whereas of 32 others born during the same period and left untreated, ten died of acute dysentery before they were seven days old.

The coliform strains most concerned in animal disease are *E. coli*, often in the mucoid phase and often atypical as to lactose fermentation, and members of the genus *Klebsiella*.

*Human pathology.* *Proteus morgani* has been reported as the etiological agent in summer diarrhea of infants, infectious diarrhea of the new-born, diarrhea and dysentery in adults, infections of the urinary tract, meningitis, chronic discharging wounds, ulcerative colitis, war wounds, fatal septicemia and a paratyphoid-like infection. Rauss (130) placed Morgan's bacillus with *Proteus*, the British System of Bacteriology (1929) put it in the dysentery group, and Winslow, Kligler and Rothberg (186) considered it a paratyphoid (*Salmonella*) which was the classification given it in the third edition of Bergey (1930), an allocation approved by Levine, Ajwani and Wedin (91), Havens and Mayfield (57), and sanctioned by the French Dictionary (56). Thjøtta (164), d'Aunoy (3), Waaler (177), and Jordan, Crawford

and McBroom (69) think of it as a coliform type because it is gas-producing, actively motile, strongly positive for indole, extremely heterogeneous serologically and distributed in a number of environments, including soil, water and normal stools (80). Since the fermentation of lactose is delayed or even absent, it seems logical to consider the Morgan bacillus as standing with the paracolon bacilli on the border of the coliform group next to the paratyphoid group, *Salmonella*.

Organisms of the genus *Klebsiella* are reported from diseases of the respiratory tract, rhinoscleroma, war wounds, suppuration, meningitis, gaseous emphysema, septicemia, fetid nasal catarrh, infections of the urinary tract, infectious diarrhea of the new-born, and bronchial asthma. The Friedländer type of coliform organism is not prominent in diarrhea, dysentery, cystitis, pyelitis, cholecystitis and cholangitis, nor is it as relatively prevalent in post mortem invasion of the body as many have supposed. Mackennon, Turner and Khayat (98) reported a study on bronchial asthma in which 28 strains of "mucoid encapsulated organisms" were studied. They confirmed the generally accepted view that such strains show very variable cultural reactions and that when present in bronchial asthma there was an associated hypersensitivity of the patients towards the intradermal test with vaccine prepared from the bacilli. The presence of gas in the tissues discovered on autopsy is usually attributed to *Clostridium welchii*, but coliform bacteria can cause this condition.

Coliform organisms not specifically labelled as *Klebsiella* or the Morgan bacillus have been reported from such conditions as pyelitis, cystitis, cholecystitis, cholangitis, suppuration, septicemia, war wounds, Winckel's disease or hemorrhagic septicemia of the new-born, sepsis neonatorum, infectious diarrhea of the new-born, gastro-enteritis, food poisoning, peritonitis, diarrhea, meningitis, arthritis, intestinal intoxication, gaseous emphysema, and rare cases of infectious dermatitis.

The importance of coliform bacteria in cystitis and pyelitis is attested to by a very large literature on the subject. The two points of chief concern to the bacteriologist working in urology are: (1) the mechanism of the invasion of the urinary tract by

bacteria; and (2) the types of coliform bacteria concerned. For two decades Dudgeon and his co-workers represented the most active group in this field (35, 36, 37). Their early work emphasized the property of hemolytic power as characterizing most of the coliform types involved, particularly in the male, and somewhat later they called attention to the slow lactose-fermenting coliform bacteria in urine. Although practically all of the atypical coliform bacteria were first isolated from feces, they were early found in urine. Thus Mair (101) described the paracolon bacillus from urine, W. J. Wilson (183) reported "anaerogenous" coliform bacteria from that source, and Kennedy, Cummings and Morrow had in their series of slow lactose-fermenters four strains from urine (78).

Hill, Seidman, Stadnichenko and Ellis (62) made an exhaustive report on the coliform bacteria isolated from cases of genito-urinary infection. They classified 200 cultures into *Escherichia*, 50 per cent; *Aerobacter*, 39.5 per cent; *Proteus*, 2.5 per cent; and miscellaneous, 8 per cent. Their data permit a breakdown of the 179 coliform strains (89.5 per cent) into *Escherichia*, 27 per cent; "coliform intermediates," 23 per cent; and *Aerobacter*, 39.5 per cent. More than half the strains were hemolytic.

Burke-Gaffney (19) studied 1000 strains of coliform organisms isolated from 126 specimens of urine. Classified by MacConkey groups 18 per cent were *B. acidi-lactici*; 7 per cent, *B. coli-communis*; 27 per cent, *B. coli-communior*; and 48 per cent, *A. aerogenes*. On the basis of indole production, methyl-red reaction and citrate utilization, 33 per cent were *E. coli*, 52 per cent *A. aerogenes*, 10 per cent "intermediates," and 5 per cent "atypical." Sandholzer (142) reported on 530 cultures of coliform bacteria isolated from 283 patients with urinary infection. Of the 530 strains 83 per cent were *Escherichia* belonging to 27 species or types, and 13 per cent were *Aerobacter* belonging to 14 species or types. The relative abundance of *Aerobacter* strains in urinary infections is striking. Hill et al. (62) reviewed the literature on the prevalence of *Aerobacter* in feces and found that among 14 reports totalling nearly 7000 cultures there were five reporting no *Aerobacter*; and in the other nine the percentage



occurrence of this genus ranged from 0.06 to 16.0 per cent. Their own data showed 39.5 per cent of *Aerobacter* in urological infections, and they stated that if the source of such infection is intestinal it is possible that the fecal organisms finding their way into the urine respond to some selective action in the genito-urinary tract which operates to favor the genus *Aerobacter* over *Escherichia* since in the bowel *Aerobacter* is far outnumbered by *Escherichia*. This point is well supported by the data of Burke-Gaffney and to a lesser degree by those of Sandholzer. If the analysis be made on the basis of citrate utilization the difference is even more marked. Ruchhoft, Kallas, Chinn and Coulter (140) summarized the findings of six workers on 2534 coliform cultures from feces. Only 9.2 per cent of these were citrate-positive. In the Hill series from urine there were 62.5 per cent of citrate utilizers. Here is indeed a nice example of the operation of ecological factors.

Food poisoning is not thought of as of coliform etiology but Buchanan and Megrail (17) in Ohio, and Gilbert, Coleman and Laviano (51) in New York have reported outbreaks apparently due to organisms of the genus *Aerobacter*. If coliform organisms can produce a toxin, as seems amply demonstrated, it is a little odd that more intoxications with this toxin have not occurred. It may well be that the human adult is relatively resistant to it.

In recent years considerable interest has been aroused by the occurrence of outbreaks of epidemic diarrhea and gastro-enteritis, apparently water-borne, but in which no definite pathogen as etiological agent can be demonstrated (176, 181, 50, 188). This problem has assumed such proportions that a special symposium on gastro-enteritis was held by the American Water Works Association in connection with its annual meeting at Buffalo in 1937 (29).

Infectious diarrhea of the new-born is a disease in which emphasis should be placed on the coliform group as probable etiological agents. This disease is highly fatal and infectious in nature and when it invades a hospital nursery it is sometimes brought under control only by closing the maternity service of the institution. The nature of the disease indicates a potent intoxica-

tion which induces a diarrhea and results in extreme dehydration. Its analogy to the clinical course of Asiatic cholera has occurred to some of the students of the problem and comparative pathology calls to mind the calf scours situation. Among recorded outbreaks Dick, Dick and Williams (33) reported *Proteus morgani*, Jampolis, Howell, Calvin and Leventhal (68), a form of *Klebsiella*; Dulaney and Michelson (38) found *B. coli-mutabile*; McKinlay (99) recovered an organism thought at first to be a paratyphoid but which was probably a coliform organism of paracolon type; and Randall (1938) encountered coliform organisms in his study of two cases.

Theobald Smith's philosophy concerning *E. coli* is pertinent at this point. He stated (152) that in the gradual evolution of pathogenic or invasive types of bacteria, the beginnings of parasitism may have been made possible by a soluble diffusible toxin, but that in later stages this primary offensive, more or less accidental, mechanism is either partly or wholly suppressed and some different mechanism developed with which the bacteria protect themselves against the body-foreign forces of the host. The process may be regarded as shifting from the destructive, predatory to the parasitic, from the offensive to the defensive type. According to this hypothesis, *E. coli* represents the early predatory toxic stage with, however, a certain specialization towards protection from anti-foreign activities in the digestive tract. It represents in many respects the cholera vibrio in its activities.

In a recent discussion on staphylococci (Levine, B. S., 1938) it was stated that probably all of the *Staphylococcus aureus* type possess the capacity to produce exotoxin. Only certain strains produce enough to induce food poisoning in man when ingested in cream puffs or other food. There is evidence that somewhat the same situation may hold among coliform bacteria.

In all considerations of gastro-intestinal disease of infectious nature one should not lose sight of the possibility that symptoms are being manifested in the bowel whereas the inciting cause is elsewhere in the body. Felsen (45) stated that the indirect hematogenous excretory mechanism of the intestine is important in explaining many poorly understood, non-specific intestinal

infections or so-called infectious diarrheas. The primary cause often exists outside the intestine, and search for specific noxious agents in the bowel is then futile. Focal intestinal symptoms often cease abruptly after the primary extra-enteric focus of infection is eliminated, but they may persist for a longer period if necrosis and ulceration have been produced. The possible rôle of upper respiratory infection on the bacteriology of the intestinal tract has recently been discussed by Lieb and Chapman (94). In cases where the intestinal manifestations are incited extraneously the bacteria of the bowel may well respond to the new conditions with an altered flora which might serve as an "indicator" (121). Sufficiently studied, such bacterial types might be resolved into instruments of diagnosis almost as surely as if they were of primary etiological significance. We have then to study infectious diarrhea of the new-born either as a locally incited disease produced in a susceptible host by toxin-producing coliform bacteria, or to consider it as of other etiology, probably viral, with the avenue of infection by way of the respiratory tract.

*Plant pathology.* In this connection it is desired merely to emphasize that the recognized coliform bacteria and the 13 species of *Erwinia*, listed in Bergey, are very closely related. F. D. Chester (1938) stated that the genera *Erwinia* and *Phytopomonas* were established on a purely utilitarian basis and have no genetic standing. Stanley (158) was of the opinion that the soft rot bacteria undoubtedly belong to the colon-typhoid-dysentery group of bacteria. Stuart, Griffin and Baker (161) studied 200 "coliform" cultures obtained from decayed portions of a number of fruits and vegetables. Serological investigations, in progress, seem to show an antigenic relationship between the plant, atypical and typical coliform organisms.

*Occurrence in the intestine.* Coliform organisms (*Escherichia* and *Aerobacter*) were first isolated from the intestinal tract of man. They were shortly recognized, though not without considerable research, as occurring in the intestinal tract of all higher animals. In examinations of meconium, commonly considered as sterile, it has been shown (22, 55, 155) that coliform

organisms may be present in a certain percentage of specimens. Throughout life, man is rarely without demonstrable coliform bacteria in his gastro-intestinal tract.

Much research has been expended in the effort to discover tests which will select fecal from non-fecal coliform bacteria (149, 18, 140, 6, 25, 122, 123, 103, 161). Certain facts emerge from this mass of data. All types of coliform bacteria may occur in feces but *Escherichia coli* (Imvic + + - -) is the most typical, numerous and constant type, with *Aerobacter aerogenes* (- - + +) next, and coliform intermediates (- + - + most common type) third. *A. cloacae*, paracoli, slow-fermenters, and *Klebsiella* may also be recovered. There is not much point in making comparisons of data unless identical methods of isolation have been used. In a certain number of cases *E. coli* may be absent, and there are even fecal specimens which yield no coliform bacteria at all (25, 123). Furthermore the coliform flora of an adult in good health and on a constant diet may show considerable change from day to day (122). When the usual fecal specimen is stored in saline suspension in the ice box considerable change occurs in the coliform flora, *E. coli* decreasing, and "intermediates" and *A. aerogenes* and *A. cloacae* increasing with, after many months, a complete change in flora often ending up with slow lactose-fermenting varieties of citrate utilizers. In about 14 per cent, however, there is no such change, the original *E. coli* persisting for months in competition with the other fecal bacteria and still presenting the characteristics of *E. coli* from fresh feces. These data are interpreted by Parr (119) to mean that in the latter case the specimens were originally pure cultures of *E. coli* so far as coliform bacteria are concerned.

The significance of these findings for sanitary science is that all of the coliform bacteria must be thought of as possibly fecal in origin. Where pollution derives from several sources one may expect to find *Escherichia coli* if the pollution be fresh; where pollution is from a single source there is no certainty that *E. coli* will be present; and the finding of typical *E. coli* may not indicate fresh pollution, particularly if that pollution be derived from a single source. Despite these qualifications, the presence of

significant numbers of *E. coli* in water remains our best test for fecal pollution.

*Occurrence in milk.* The recent literature on coliform bacteria in milk is even more voluminous than that for these organisms in feces (81, 44, 102, 182, 6, 7, 187, 10, 115, 162). Stark<sup>10</sup> has stated that, due to what we may call the "living conditions of cows," most raw milk contains coliform bacteria. These organisms in milk are assumed to come from barnyard manure, and since cows do not have typhoid fever, the presence of coliform bacteria in raw milk is known not to be of the same public health significance as is their presence in water. They are uniformly regarded as undesirable bacteria to have in milk and dairy products because they produce gas and undesirable flavors and odors. Their significance is largely proportional to the numbers present. It is important to remember that, unless some inhibiting condition is present, these bacteria grow well in milk. Although bacteria belonging to this group are occasionally found able to resist the heat treatment of the pasteurizing process, their presence in pasteurized milk is usually interpreted to indicate recontamination. The seriousness of permitting pasteurized milk to become recontaminated with any kind of bacteria is readily recognized. The pasteurizing processes applied to cream for buttermaking and ice cream mixes are generally accepted as adequate to destroy coliform bacteria. Their presence in these products is also believed to indicate recontamination of a pasteurized product. The types of coliform bacteria present in milk will vary with the flora of the feces, soil, or grain dust contaminating it.

*Occurrence in soil.* Coliform organisms are common in soil. Minkewitsch, Rabinowitsch and Joffe (108) believe that these bacteria are not found in the soil where there is no animal life. As pointed out by Thom (1938), it has been assumed that the presence of the colon group in soil is due to fecal contamination, and for that reason coliform bacteria have not particularly engaged the attention of soil microbiologists. In soil *A. aerogenes* is more abundant than *E. coli*, and "intermediates" and atypical

<sup>10</sup> Coliform Round Table, 1937.

forms are present. This picture will vary with the character and use of the soil from the *E. coli*-sparse, virgin, protected soil to the *E. coli*-rich pasture grazed over by animals. From some quarters there is evidence that some, at least, of the citrate utilizers and atypical forms in soil are derived from fecal *E. coli* and typical intestinal forms. Minkewitsch, Rabinowitsch and Joffe (108) report the change of *E. coli* seeded in soil into citrate-utilizing "intermediates." This is far from the production of *A. aerogenes* from *E. coli* which, so far as we are aware, has never been reported, but it does indicate a step in coliform evolution. Parr (124), working with one of Koser's original soil strains, V5, in laboratory cultivation for more than a decade, has derived *E. coli* (+ + - -) from the strain called originally an "intermediate" (+ + - +). Despite the fact that citrate utilizers predominate there is evidence that *E. coli* can survive for a considerable time in soil (121).

*Occurrence in urine.* The striking thing in urine, as in soil, is the shift in the coliform picture from what it is in fresh feces to a predominance of citrate-positive coliform bacteria (62, 19, 142). The same shift occurs, as we have shown, when the usual fecal specimen is stored. The mechanism of these shifts may be a matter of variation, but is more likely succession, conditioned by ecological factors.

*Occurrence in water.* The literature of water bacteriology is much too complex to be reviewed here, as it touches on mediums, tests, interpretations and standards (139, 140, 18, 52, 169, 24, 48, 8, 60, 128, 14). Personal communications (1938) from Kulp, Levine, Norton, Butterfield, Mickle, McCrady, Norcum and G. F. Edwards have called attention to many sanitary problems connected with the coliform bacteria. Kulp feels that an attempt should be made to differentiate between *E. coli* and *A. aerogenes* especially when dealing with private water supplies, whereas Butterfield states that it is his policy to attach equal sanitary importance to the presence of each member of the coliform group since all are found in feces and since they are about equally susceptible to the forces of natural and artificial purification processes. Norcum is concerned over the increasing prevalence

of gastro-enteritis, apparently water-borne, but with questioned etiology. Norton mentions the significance of the work of Heathman, Pierce and Kabler (60) from which it appears that *E. coli* may be no more (or even less) resistant to chlorine than the typhoid bacillus, and he feels the chlorine resistance of coliform bacteria should be restudied. McCrady is also anxious to have the significance of atypical coliform bacteria cleared up, and the New England workers are particularly concerned over the necessity for generous interpretations of standards to avoid condemning too many water sources epidemiologically satisfactory. Coliform bacteria do not occur ordinarily in water except from contamination with soil washings and fecal material from man and animal. When the pollution is from feces these bacteria survive for some time but generally with a shift from citrate-negative predominance to citrate-positive predominance. There are, however, both theoretical and actual conditions under which *E. coli* may persist with typical reactions for long periods of time. Usually, though, the numbers of coliform bacteria decrease and in the absence of recontamination the group is usually lost sight of after a few weeks.

*"Pump infection," and paper and wood pulp.* It is well established that coliform bacteria grow well on leathers and other organic pump-parts, on swimming-pool ropes, and in pipe slime (24, 87, 1, 144). The growth of these bacteria in water distribution systems, of course, affects the analysis of the water. L. S. Stuart (1938) has reviewed the bacteriology of the tanning process from which it is apparent that modern leather is not itself the source of these coliform growths. The forms which are likely to occur naturally are "intermediates" and *A. aerogenes*, but other organisms will grow on jute and leather (*Serratia*, *Escherichia*, and even the typhoid bacillus).

The part played in the paper and wood pulp industries by coliform bacteria is but seldom mentioned. Tonney and Noble (168) have noted the persistence of *E. coli* and *A. aerogenes* on wood. The quality of water in contact with wood may be impaired by a high coliform count under conditions somewhat analogous to pump "infection." In 1931 Beckwith (11) re-

ported on the bacteriology of pulp slime and pointed out the importance of *A. aerogenes*. In 1938 he stated that it was his opinion that pulp slime has as one of its important causes the growth of capsular bacteria, nearly all of which are coliform. He showed, with Morgan, that the mucoid type appears frequently if the incubation temperature is low, and, of course, in the presence of a certain amount of carbohydrate. In "white water" the temperature is low, and it frequently contains appreciable amounts of carbohydrate which possibly are produced by inversion of the cellulose. Sanborn (1938) indicated the need for a detailed study of the coliform organisms found in pulp and paper mill systems and stated that he had seen pulp wood logs coated with gelatinous slime due to the development of organisms related to the genus *Aerobacter*. Chlorination and the high temperature of drying eliminate the bacteria so there is but little danger of the spread of bacteria by paper containers; but pulp containing slime organisms works up into defective finished products so that the problem is one of economic importance.

*Olives.* Alvarez (2) studied the blister-covered olives commonly called "floaters" and found that the condition was caused by atypical organisms "closely allied to, but not identical with the colon group." One strain, "H," resisted 80°C. for 45 minutes. Ten per cent salt solution was required to kill it in 24 hours. Again, Tracy (171) emphasized the spoilage of olives by colon bacilli. The reviewer has been given to understand that the coliform group constitute the most important olive spoilage organisms and that recoveries are mostly "intermediates" and *Aerobacter*, but occasionally *Escherichia*.

*Shellfish.* The presence or absence of fecal pollution in oysters and mussels is determined by examination for coliform bacteria (13, 126). In 1938, Perry stated that the examination of shellfish and their growing waters cannot be considered in the same category with drinking water which can be filtered, chlorinated or protected. Perry holds that many coliform bacteria, particularly of the *E. cloacae* type, are present in shucked market oysters or shell oysters when the temperature exceeds 60°F., that they are without significance as indicating pollution, and



that *E. coli* is the logical indicator of fecal pollution in shellfish and shellfish growing waters.

*Foodstuffs; miscellaneous.* It would appear from the nature of the processing procedures involved that canned foods do not contain coliform bacteria, a surmise confirmed by Williams (1938). Crossley (30) found 88 per cent of 14,365 samples of meat and fish pastes sterile, with coliform bacteria having small significance among the positives. In other types of foods their importance is greater as shown by the report of Griffiths and Fuller (53) on the detection and significance of *E. coli* in commercial fish and fillets, and that of Hunter (65) who found coliform bacteria important in salmon spoilage. One of the "believe it or not" of bacteriology is the record of Simonds (148) that in a World War depot in Belgium three barrels of soft soap exploded due to growth of bacteria of the genus *Klebsiella* in the soap. The work of Burkey (20) on the fermentation of corn stalks and their constituents by bacteria of the genus *Aerobacter* has further extended our appreciation of the ubiquity of coliform bacteria. Lastly, Minkewitsch (107) has pointed out the part that insects play in the spread of coliform bacteria in the soil and on plants.

#### ATYPICAL COLIFORM BACTERIA

The significance of atypical coliform bacteria was early recognized, for in 1899 a committee composed of Veranus A. Moore, J. G. Adami, Elmer G. Horton and J. Monjares, was appointed by the section of bacteriology and chemistry of the American Public Health Association to study variations of the colon bacillus in relation to public health.

For convenience we may divide the atypical coliform bacteria into two classes. There are, first, those forms which give most of the reactions peculiar to a particular species but differ from it in some slight degree not sufficient to be named as another species. Such, for instance, are chromogenic *E. coli* (116, 120, 167, 161); encapsulated *E. coli* (153, 118); the sugar-tolerant coliform organism described by James (67); the organism giving common

serological reactions at high titre with the *Salmonella* (54); *A. transcapsulatus* (163), in which the organism lies at right angles to the greatest diameter of the capsule; *E. coli* with polar flagella (66); a heat resistant form (2); the organisms which are methyl-red positive and also Voges-Proskauer positive, or "double negative"; gelatin-liquefying *E. coli*; cellobiose fermenting *E. coli*; and hydrogen-sulfide positive *E. coli*. The property of hemolysis is hardly an atypical feature for it is common to many strains of *E. coli* both from the urine and the bowel. Such atypical forms are confusing to the taxonomist but probably not as much so to sanitarians as the second class of atypical coliform bacteria.

In the second category we place the instances of fermentation irregularities encountered in these bacteria. It will suffice to consider only irregularities encountered with lactose. If one seeds a tube of lactose broth with a typical coliform organism, within 24 hours full acid and gas production will appear. In water analysis, a positive tube must show acid and gas production within 48 hours. What about the tube which has acid and only a bubble of gas in 24 hours but never any more, or one which has full gas production but requires 72 hours to produce it? These are the organisms concerning which McCrady (1938) circularized workers interested in coliform bacteriology.

Moreover, coliform organisms are frequently encountered which fail to ferment lactose for a considerable number of days. Such strains are often confused with paratyphoid bacteria. They are the true slow fermenters and in many cases can be trained to rapid fermentation. Many of them are *Bacterium coli-mutabile* or mutabile types of *Aerobacter* and as such appear to be unstable variants as described by Deskowitz (32), earlier called "mutants."

Other atypical forms are those strains which ferment lactose producing acid but failing to produce gas. For these the term "anaerogenous" is used. Again there are strains which give all of the reactions for *Escherichia* except the fermentation of lactose and which fail to give serological reactions with *Salmonella*.

These are called "paracoli" and may not ferment lactose no matter how long cultured. One further variant is the strain that ferments lactose at room temperature but not at 37°C.

The prevalence of such strains is indicated by Malcolm's work (103) with 1636 cultures of which 3 per cent were atypical. Kline (81) found 126 "anaerogenous" *E. coli* among 325 cultures isolated from raw and pasteurized milk. He expressed the opinion that these organisms are really members of the colon group which may have become modified through the influence of an unfavorable environment. We believe the evidence warrants the view that the slow fermenters, the "anaerogenous" strains, Morgan's bacillus and "paracoli" strains are all coliform bacteria which may be placed with whichever species they have the most characters in common.

It is much more difficult to assess the significance of the slow fermenters and other atypical coliform bacteria. It has been suggested that there is some relationship between the power to ferment lactose and virulence, as shown by the fact that the pathogens of the colon-typhoid group do not ferment lactose, and also by Dudgeon's (37) account of 49 cases of very severe acute infection of the genito-urinary tract in which all the strains of *B. coli* showed delayed fermentation of lactose. It has been found that atypical forms are likely to occur in stool specimens from subjects showing evidences of gastro-intestinal ill health (47). If these points of view be true, it would seem that the atypical strains encountered in water analysis should have more significance as indicators of dangerous pollution from feces or urine than more typical strains. Difficulty arises from the fact that atypical strains are also found in many environments in which coliform bacteria without pathological significance survive. This is known to the water bacteriologist who is inclined to look upon slow lactose-fermenting coliform organisms as "attenuated" or "devitalized" forms.

#### VARIATION IN THE COLIFORM GROUP

The "unstable variant" is by far the most interesting of bacterial variants. *Bacterium coli-mutabile* (113, 104) is a good

example. When this organism is cultured on lactose indicator-agar it appears not to ferment lactose. After some days, however, papillae appear growing on or out of the original colonies. Sub-cultures from these secondary colonies give typical lactose fermentation but sub-culture from the primary colony, avoiding contact with the papillae, gives delayed fermentation and will, when again plated, reproduce the original picture of colonies, negative to lactose, but on which lactose-fermenting secondaries eventually appear. One may take such a strain and plate it serially hundreds of times. It will still produce non-fermenting colonies on which fermenting papillae later appear. Such strains Deskowitz called "unstable variants." The early workers (113) thought of them as de Vriesian mutations, Stewart (159) attempted to explain them on Mendelian principles, and Mellon (105) has considered *Bacterium coli-mutabile* as a transitional developmental stage between the normal strain of *E. coli* and wild, non-lactose-fermenting *E. coli*. Such "unstable variants" are not uncommon and their peculiar type of variation is manifested in changes in colony type as well as in biochemical reactivity. Thus Deskowitz was working with the R-S colony type variation as manifested by certain strains of *Salmonella aertrycke*; and what appear to be "unstable variant" phenomena are recorded by Koser and Vaughan (86) in their paper on the utilization of d-arabinose by bacteria. It is possible, also, that the citrate "mutant" described by Parr may be another instance of "unstable" variation. It should be stated that except in the case of capsulated forms the discussion deals, so far as the records show, with smooth phase cultures.

Recent work with variations in the ability to ferment sucrose have interested workers in the coliform field and challenged taxonomists. Sherman and Wing (146) found that certain recently isolated strains of *E. coli* and *A. aerogenes*, seeded in pure culture in salicin and sucrose broths, gave rise to progeny which varied from the parent strains used. For example, from a culture of *E. coli*, which fermented salicin but not sucrose, progeny of four fermentative types were obtained which would by some terminologists be named as four different species. Treg-

oning and Poe (172) confirmed the production of sucrose variants, whereas Fulton (49) was unable to do so. Minkewitsch, Rabinowitsch and Joffe (108) have also reported the production of sucrose-fermenting strains from sucrose-negative antecedents. There seems to be marked difference in the facility with which strains of these bacteria vary and the frequency of appearance of strains capable of variation. Thus in our work we encountered up to the summer of 1938 only 29 instances of coliform strains giving the citrate "mutation." But, June 2, a fecal specimen was examined in which 54 of 60 colonies, picked, purified and studied, were *E. coli* which gave off in each instance small numbers of variants that one would have to classify as atypical *E. freundii* since they were citrate-positive and hydrogen-sulfide negative.

Nyberg, Bonsdorff and Kauppi (114) reported that two of their strains changed from *Escherichia* to *Aerobacter*. This statement was made on the basis of a change from M.R. + V.P. - to M.R. - V.P. + after isolation. Citrate was not used in this work. Such changes were observed by Koser (84) who reported eight soil coliform strains which reversed their methyl-red and Voges-Proskauer reactions. Koser's cultures were, however, all positive utiliziers of citrate so that the change observed was not from *Escherichia* to *Aerobacter* but a shift of type within the "intermediate" group. Minkewitsch, Rabinowitsch and Joffe (1936) reported changes *in vitro* and in the soil of *E. coli* to "intermediates." On the basis of their findings they suggested that it might be argued that all coliform bacteria arise from fecal *E. coli*. Most workers, however, seem to feel that the direction of evolution in the coliform group has been from the highly reactive, ubiquitous *A. aerogenes* to the less reactive, more specialized parasitic types.

Passing over numerous interesting references to the alteration of cultural finding in the coliform group through the use of chemicals, immune serum, and the like, we next discuss "shifts." Nyberg, Bonsdorff and Kauppi (114) in 1935 studied 200 cultures isolated at Helsingfors in 1933. They found that 68 strains were not viable and that only 25 of the 132 viable cultures had the

original colony type and cultural reaction. Fifty-nine cultures had changed in both colony type and reaction and 11 more, although retaining the original colony type, gave different reactions. Stuart, Griffin and Baker (161) studied "shifts" in the reactions of 191 cultures and found that in 47 instances changes occurred. They suggest that it might be better to use the term "stabilization" rather than "purification" for the treatment to be accorded cultures, and hold that "purification" implies contamination by a foreign species whereas "stabilization" implies a reasonable constancy of reaction without excluding the possibility of variation under suitable conditions.

#### SEROLOGY

Van Loghem (173) early emphasized the serological heterogeneity of the coliform group, stating: "Das individuelle Benehmen der Coli-Bazillen bei serologischen Untersuchungen ist bekannt. Stellt man ein Immunserum her mit einem bestimmten Coli-Stamme, dann findet man selten andere Coli-Stamme, welche von diesen Serum agglutiniert werden." Even fecal strains isolated from the same plate will not generally be influenced by the antisera prepared from any of the others. There are two possibilities. Either the number of kinds of *E. coli* is very considerable or the serological variability is very great. Such extreme variability could only be conceived of on some such theory of antigenic flux or instability as van Loghem had in mind: "der Rezeptorenapparat des *B. coli* sich in einem Zustande stätiger immer Verschiebung befindet, so dass sie Characterzüge, welche bei anderen Bakterien die spezifischen Serumreaktionen ermöglicht haben, bei Coli-Bazillen bald wieder ausgewischt werden."

Mackie (100) stated that, while an immune serum to a particular strain of typhoid bacillus will agglutinate most strains of *B. typhosus* with but little variation in degree, immune serum to certain *B. coli* types, on the other hand, have been found to exert little or no action on other strains identical as regards cultural reactions with that used for immunization. Smith (154) said: "The relation between a strain of *B. coli* and its mutant

with reference to the production of agglutinins and protective antibodies may be expressed by the statement that the original strain when injected into cows develops antibodies both toward itself and the mutant, whereas the mutant produces them only towards itself." The citrate "mutant" reported by Parr (124) reacted to the same titre as the parent strain with a serum prepared against the parent. On the other hand, Sievers (147) reported that a coliform strain gave two variants, a gas former and a strain which did not form gas. Sera were prepared from both variants and these sera were not identical. Havens and Irwin (58) also observed an antigenic change coincidental with the acquisition of sucrose fermentation in the Morgan bacillus, "no cross-agglutination" occurring between the sucrose-fermenting and the non-sucrose-fermenting "strains from the same culture."

Lovell (95), employing the precipitin test, found that 79 of 110 strains of coliform bacteria from diseased calves fell into eight groups. Hitchener (1938) prepared sera against eight slow lactose-fermenting strains and tested 19 cultures of these organisms against the eight sera. Four strains were encountered which fell into one group but the others were individualistic. It is true that Dudgeon, Wordley and Bawtree (36) found that most of their hemolytic strains of coliform bacteria isolated from acute urinary infections were agglutinated by a serum prepared from one of them. However, the non-hemolytic cultures from the same source showed no such relationship.

Serological work with coliform bacteria of the genus *Klebsiella* has, however, led to more satisfying results. In 1926 Julianelle (71, 72, 73) established the fact that the specificity of these organisms resides in their capsular materials. He studied a series of Friedländer bacilli and classified them into three specific types A, B, and C, and a heterogeneous group X. Edwards (40) tested 50 strains of encapsulated bacilli and was able to place 43 in two serological groups, seven remaining untyped. In 1929 he found that five cultures of *Bact. aerogenes* were serologically identical (as then tested) with type B Friedländer bacilli and two were identical with a strain of the *granuloma bacillus*.

In 1934 Morris and Julianelle studied rhinoscleroma strains and found them serologically identical with type C Friedländer bacilli. Barnes and Wight (9) studied a hemolytic, encapsulated strain of *E. coli* which appeared to have antigenic identity with pneumococcus type I. In 1937 Julianelle (75) examined strains of *Bacterium aerogenes* and showed there were three type-specific immunological entities among them, one common to pneumococcus type II, one common to both pneumococcus type II and Friedländer's bacillus type B, and a third which was individualistic. He also showed that strains of *Bacterium aerogenes* differ serologically when encapsulated, but become antigenically the same when de-capsulated. This leads us to the most important recent advance in the serology of the coliform group. Studies by Julianelle (76) on the immunological reactions of the unencapsulated cell supplied the hypothesis that the different organisms once deprived of the ability to elaborate capsular polysaccharide might be more readily amenable to systematization. Accordingly, unencapsulated, or "R" strains,<sup>11</sup> were derived from the encapsulated "S" strains by continued cultivation of the "S" form in homologous anti-S serum.

With such unencapsulated strains and such sera, Julianelle studied some of the encapsulated coliform bacteria (No type B Friedländer bacilli were included) and found that they fell into two main groups: one including Friedländer bacilli types A and C; and the other rhinoscleroma, ozaena, *A. aerogenes*, and granuloma strains. More recently (1938) Julianelle has tested three strains of *E. coli* and two of *A. aerogenes* in a preliminary study. The former fell into two groups and the latter into one. Such serological work is laborious and time-consuming, but it is possible that some such approach as this will prove very fruitful.

#### CLASSIFICATION OF THE COLIFORM BACTERIA

Malcolm (103) has stated that the coliform group of bacteria consists of a gradation of types so closely linked together as to render it undesirable to divide the group into two genera. The

<sup>11</sup> The designations of the culture phases are those of Julianelle (76).



reviewer hopes to convey a concept of coliform bacteria as a group of closely related, closely intergrading bacteria in which, by the dropping of one character or the acquisition of another, an organism appears as a new strain. It is only reasonable to suppose that at intervals along the gamut of numerous varieties one can pick out a strain that will differ in a number of respects from another selected from another locus in the series.

The error of past classifications has been to dignify each recognizable variety encountered with a name. We now know that some of our most cherished measuring rods, such as the methyl-red and Voges-Proskauer reactions, sugar fermentations, indole production, and the utilization of citrate as a sole carbon source, are not to be depended upon to give with the same organism at all times the same reaction. Coliform bacteria are particularly restless when compared with most other groups of bacteria. It is among them that the most interesting and numerous instances of variation, and "shifts" occur.

The most fundamental objection, from our point of view, to the establishment of more than one genus for the coliform bacteria is that to do so will obscure for all save a few who are unusually conversant with the group its essentially intergrading nature. The correct orientation and stimulus which this point of view provides should result in further needful research in the field.

It seems, furthermore, that the concept of the coliform "intermediates" is such that if we are to have more than one genus we must also recognize one for the "intermediates." To classify these forms with *Escherichia* is to obscure the significant points that characterize "intermediates" as such, and to lose sight of their essential intermediate nature.

For some time we have regarded *Klebsiella* as coliform strains derived from the more definitely recognized types of the group, such as *Aerobacter*, and differentiated from them by ecological factors to manifest a lessened and variable biochemical activity, a more distinct encapsulation and, in some instances at least, enhanced virulence. Serology indicates their close relationship to *Aerobacter*. *Aerobacter* is less a toxin producer than is *Escherichia*, and it is more easily degraded by environmental

influences. Such a form as Friedländer's bacillus is, according to Smith, a more advanced or developed pathogen than the toxin-producing *E. coli* which Smith speaks of as "a primitive aggressive form."

If the Friedländer bacillus has evolved beyond the primitive toxin stage, as conceived by Theobald Smith, it would require some sort of specialization in order to maintain its position successfully as an invader of respiratory membranes. This the capsule supplies. It would seem that there is little to be gained in setting up a number of species in this group based on very little else than host source.

Personally, we should like to follow Jordan (General Bacteriology, 11th edition, 1935) in retaining the genus *Bacterium* for the entire group of coliform bacteria. It seems, however, that there are taxonomic difficulties preventing this. For a genus name to be valid its type species must be recognizable, and *Bacterium triloculare* Ehrenberg, 1828, is of course unrecognizable. *Bacterium* was retained for some time as a temporary genus but it is now felt that the time has passed for a continuation of such a status (15). Were the taxonomists to propose one genus for all coliform bacteria, it is our suggestion that it should comprise the two species, now called *E. coli* and *E. freundii*; the two now designated as *A. aerogenes* and *A. cloacae*; and one of the six now listed under *Klebsiella*, presumably *K. pneumoniae*.

#### CONCLUSION

The coliform group of bacteria has presented distinct problems in classification in the past. As early as 1893 Denys and Martin (31) indicated the two main reasons for the encumberment of bacteriology with false species as, first, a paucity of comparative studies, and second, the variations to which a single species is subject. Sufficient data have now accumulated to warrant revision of former classifications. An understanding of the complexity of the group as revealed by the application of a wide variety of biochemical tests and an appreciation of its variability and changeability has convinced workers of the futility of attempting to give species rank to more than a few of the many

types that have been described. This point of view has been strengthened by the apparent failure thus far to demonstrate that certain strains are peculiar to a given environment or activity. It appears rather that coliform bacteria must be thought of as a well-nigh ubiquitous group of organisms. Of these, that

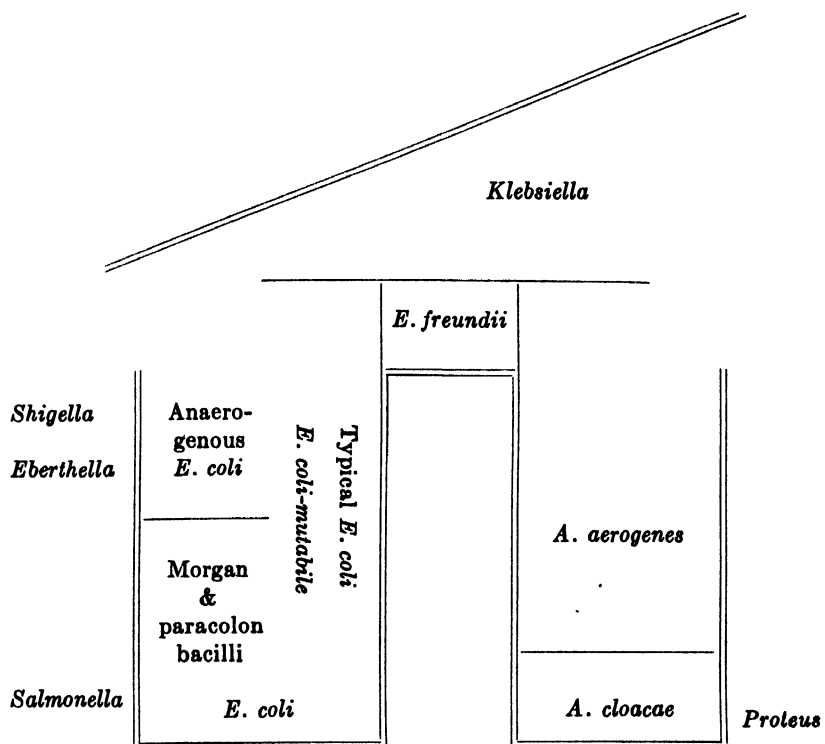


FIG. 1. A CONCEPT OF COLIFORM CLASSIFICATION

type will predominate in a given environment which is best adapted to the conditions of living found there.

The recent revision of Bergey's Manual of Determinative Bacteriology (12) has wisely taken cognizance of these principles and radically revised coliform classification in the direction of simplicity. The opportunity which this review offers for comment on that classification has been taken advantage of and suggestions have been made for further progress which our ac-

quaintanceship with the organisms and their literature would seem to warrant.

The coliform group is a large one made up of closely related, highly intergrading, and somewhat unstable bacteria which form a fairly wide gamut or *continuum* extending from the lactose-negative paracolon forms at one extreme to the highly reactive *A. aerogenes* at the other. Standing with the paracolon forms next to *Salmonella*, one finds the Morgan bacillus. In about the same position, and leading to *Eberthella* and *Shigella*, are located the anaerogenous *E. coli*. Next to these varieties come the slow lactose-fermenting *E. coli* so likely to be manifested as "unstable variants." Completing one side of the picture, one finds the typical *E. coli* which bridge over to the *A. aerogenes* side by way of the "intermediates." Below *A. aerogenes* we find *A. cloacae* which appears to point toward the genus *Proteus*, and above all forms, but particularly above *A. aerogenes*, are located the Friedländer organisms. We have tried to represent this concept graphically in figure 1.

It is felt that our present understanding of the coliform group requires for its best expression the allocation of all these bacteria to five species within one genus.

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# QUANTITATIVE ABSOLUTE METHODS IN THE STUDY OF ANTIGEN-ANTIBODY REACTIONS

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The first comprehensive theories of antigen-antibody interaction were due to Ehrlich and to Bordet. The emphasis of Ehrlich's theory was placed on the chemical reaction of antigen with antibody, while that of Bordet's was laid on the adsorption of one by the other. Bordet's own modification of his theory and the formulation by Arrhenius and Madsen of the Ehrlich view in terms of reversible reactions which could be expressed on a mass law basis still left a wide gap between the theories. This gap was not bridged or accounted for by the commonly adopted use of colloid chemical terminology. In more recent years, however, adsorption has come to be regarded as a chemical, rather than a physical process, so that the old distinction between the Ehrlich and Bordet theories has become scarcely more than a point of view. The demonstration by Proctor and Wilson and especially by Jacques Loeb that the laws of classical chemistry could be applied to the behavior of typical colloids such as proteins has given direction and force to subsequent studies in innumerable fields of biological research, and in particular to



the studies on the mechanism of immune reactions on which most emphasis will be placed in this review.

At the time the Ehrlich and Bordet theories were formulated, and for many years afterward, little was known of the chemical nature of either antigens or antibodies. The fundamental researches of Landsteiner (1), the discovery of the immunologically specific polysaccharides by Avery and Heidelberg (2) and the ensuing work on artificial carbohydrate-protein antigens by Avery and Goebel (3), and many other studies which have been reviewed elsewhere (1, 4, 5, 6, 7) have led to a better understanding of the relation of chemical structure to antigenic function. As to antibodies, it is generally conceded that these are actually modified serum globulins and a considerable body of information as to their properties is already available (4-7). A modern theory of antigen-antibody interaction will therefore not only rest on a more secure understanding of the nature of the substances involved, but must also account satisfactorily, and in the last analysis quantitatively, for a very large accumulation of knowledge.

A cause of early interpretative difficulties in the study of antigen-antibody interactions, promptly recognized by Ehrlich and Morgenroth (8), was the common use of whole sera or other multiple component protein mixtures as "antigen." A few investigators, however, did not encounter these difficulties, and drew conclusions of far reaching importance. Owing doubtless to the preponderance of casein in milk the antisera obtained by Müller on injection of milk into rabbits (9) seem now to have behaved like antisera to a single antigen, and yielded a precipitin reaction zone in which neither antigen nor antibody could be detected in the supernatant. An eight- to ten-fold range of combining proportions was noted and evidence given of an actual soluble antigen-antibody compound in the non-precipitating region of a large excess of milk. Similarly, a four-fold zone of combining proportions in which neither component could be detected in the supernatant was observed by von Dungern (10) in studies on the precipitin reaction in rabbit antisera to octopus, crab, and mollusc plasmas. By the demonstration of hemo-

cyanin in the specific precipitate by the blue color and its reversible disappearance and reappearance with carbon dioxide and air, von Dungern was the first to show the value of the "marked antigens" which later were to figure so prominently in the study of the precipitin reaction. He also emphasized the presence of several distinct antibodies in the sera studied and commented on them in terms of the reactive groupings involved.

Although the broad range of combining proportions in antigen-antibody reactions had already been stressed by Danysz in explanation of the effect which he discovered in toxin-antitoxin interaction (11) and was later cited by Fleischmann and Michaelis in pointing out the fallacy of precipitin measurements by volume (12), these fundamental observations were neglected by Arrhenius and Madsen (13) in their comparison of antigen-antibody reactions with the union of weak acids and weak bases.

Until recently the only analytical methods available for the study of antigen-antibody interactions were either those based upon biological effects, with their large variation in individual animals, or the purely relative serological dilution methods, which, owing to their large capacities for subjective and other errors, have remained to this day essentially qualitative in spite of cumbersome precautions. Important steps toward the solution of analytical chemical difficulties in the study of the precipitin reaction were taken by Wu and his collaborators (14). The hemoglobin (Hb)-antibody and iodo-ovalbumin-antibody systems were studied. In the former, Hb, another "marked antigen," could be determined colorimetrically in the washed specific precipitate. Total nitrogen was also estimated by the Folin-Wu modification of the micro-Kjeldahl method, the two analyses affording a means of analyzing for both antigen and antibody in the precipitate. Although two analytical principles upon which later progress was made were thus laid down, the conclusions drawn from these obviously only tentative experiments were at variance with earlier and later well-founded observations and Wu unfortunately published nothing further along these lines.

## QUANTITATIVE STUDY OF THE S III-ANTIBODY SYSTEM

In the meantime the writer had begun a study of the precipitin reaction and had found a modification of the Pregl micro-Kjeldahl nitrogen method both convenient and accurate. The study was then continued over a number of years, largely in collaboration with F. E. Kendall. The analytical difficulties were in part overcome by the use as "antigen" of the specific polysaccharide of Type III pneumococcus, the salt of a nitrogen-free polyaldobionic acid (15) which had been obtained in a state approaching analytical purity.<sup>1</sup> The analytical problem was further simplified by the use of partly purified antibody in the form of solutions prepared according to Felton (17) from Type III antipneumococcus horse serum. After "ageing" or stabilization, roughly one-half of the nitrogen in these solutions was found to be specifically precipitable by S III. Since the polysaccharide added in varying proportions contained no nitrogen, the difference between the original N content of the solution and that remaining after centrifugation of the precipitate gave an accurate measure of the precipitated antibody in absolute weight units instead of in the relative terms then customary (18). When it was found that identical results were obtained by direct analysis of the washed specific precipitate and that the amount of antibody-N precipitated was independent of the non-specific N or protein present (19-22) the more cumbersome measurements by difference were abandoned.

It was found that when a very small amount of S III was added to a relatively large amount of antibody, A, more than 240 mgm. of A might be precipitated for each milligram of S III. When increasing amounts of S were added to separate portions of A the ratio of S to A in the precipitate increased steadily, with no evidence of discontinuity. In this region of the reaction range no S could be found in the supernatant by the delicate serological test sensitive to S in dilutions of 1:10,000,000, so that it seemed

<sup>1</sup> Subsequently referred to as S III or S. All but the studies on rabbit sera described in a later section were carried out with polysaccharide preparations now known to have been degraded by heat (16). This influenced only the numerical values obtained, not the conclusions based on the data.

reasonable to assume that all of the S added was in the precipitate. In this region antibody was still in excess, as was shown by the addition of a little S to the supernatant. When still larger quantities of S were added to the same amount of A a region of the reaction range followed in which neither S nor A, or only a minimal amount of each, was demonstrable in the supernatant from the precipitate. We have termed this region, often of considerable extent, the "equivalence zone." With still larger amounts of S, the latter finally appeared in the supernatant, and in this region precipitation of antibody remained at a maximum while more and more of the S added entered into combination until in some sera constant composition was attained. Finally, excessive amounts of S III caused the formation of less and less precipitate in an "inhibition zone," until precipitation was entirely prevented. The reaction course, except for the inhibition zone, is illustrated by the curves in figure 1.<sup>2</sup> It had previously been found (21) that such reaction curves, up to the equivalence zone, could be expressed by the empirical equation

$$\text{mgm. antibody N precipitated} = a S - b S^2 \dots \dots \dots [1]$$

These reactions were found to be reversible in the sense that the precipitate formed in the region of excess antibody took up S when shaken with a solution of the polysaccharide and even dissolved in concentrated S solutions. The reversible shift in composition of the hemocyanin precipitate in either direction with antigen or antibody had been shown long before by von Dungern (10). It therefore seemed reasonable to postulate the following equilibria in the four limiting regions of the reaction range: at extreme antibody excess,  $S + 4A \rightleftharpoons \underline{SA}_4$ ; at the mid-point of the equivalence zone,  $S + A \rightleftharpoons \underline{SA}$ ; in the antigen excess region,  $\underline{SA} + (x - 2)S \rightleftharpoons \underline{S_{x-1}A}$ ; and in the inhibition zone,  $\underline{S_{x-1}A} + S \rightleftharpoons S_xA$ . The underlined formulas represent precipitates, and in all formulas the composition is expressed in arbi-

<sup>2</sup> This figure was also used in an article on "Chemical Aspects of the Precipitin and Agglutinin Reactions" read before the American Chemical Society's Symposium on the Physical Chemistry of Proteins at the Milwaukee Meeting, Sept., 1938; Chemical Reviews, 1939, 24, 323.

trary units, not molecules. In the first two equations equilibrium must lie far to the right as measurable dissociation could not be detected. It was shown that  $S_xA$  contained one more molecule of  $S$  than the precipitate with which it was in apparent equilibrium (18), confirming the belief of Müller (9), von Dungern (10), and Arrhenius (13) in a soluble antigen-antibody compound

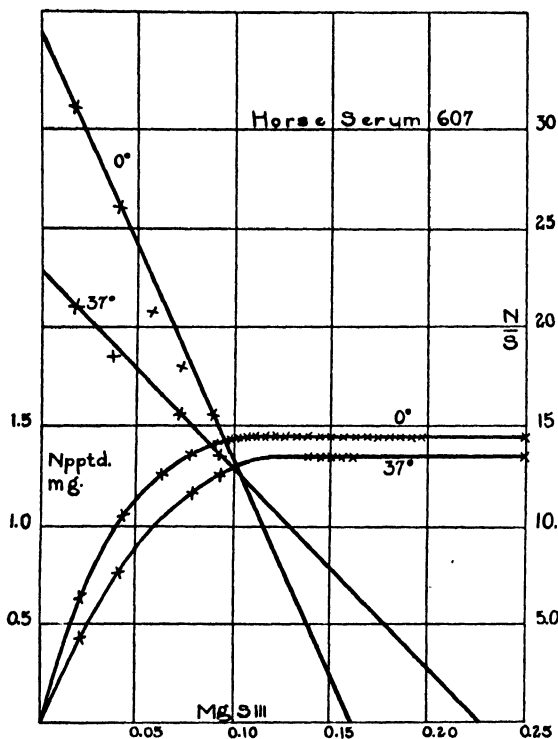


FIG. 1.<sup>2</sup> Curves: Antibody N precipitated from antipneumococcus Type III horse serum by increasing amounts of S III. Lines: Ratios of antibody N:S III in precipitates, over range indicated by crosses.

in this zone, rather than the obscure "peptization" of the precipitate which had been proposed by advocates of the colloidal theory despite Müller's direct chemical evidence to the contrary.

Since S III is the highly ionized salt of a polymeric aldobionic acid (15), and antibody globulin, dissolved in physiological media, probably exists as an ionized sodium chloride complex, the initial reactions, at least, may be ionic. The application of

the mass law in some form would seem justified. The precipitin reaction between S III and homologous antibodies would then be merely a complex instance of a specific precipitation such as that between barium and sulfate ions or silver and cyanide ions. Even the inhibition zone would have at least a partial analogy in the well-known solubility of silver cyanide in excess cyanide solution.

In qualitative terms this interpretation of the precipitin reaction appears satisfactory, but difficulties arise in the quantitative formulation of the reaction in terms of the law of mass action. It might, for example, be expected that there would be definite steps or stages between the limit of  $SA_4$  in the region of large antibody excess and  $SA$  in the equivalence zone, also in the inhibition zone, but such abrupt changes in composition are not found. This might be explained by assuming a continuous series of solid solutions, or that the mutual multivalence of S and A is so great as to permit formation of a continuous series of compounds. There are, however, valid objections to these views in spite of the well-founded structural (15) and other evidence (24) that S III contains a number of recurrent immunologically reactive groupings or valences, and the present-day views as to the structure of proteins (25), which are in entire accord with the assumption of recurrent groups of amino acids which might be "valences" or the centers of specific combination. The principal difficulty in the formulation of the reaction along these lines lies in the finding that the composition of the precipitate depends upon the proportions in which the components are mixed, and not upon the antibody concentration at equilibrium, or at the end of the reaction (23). This remarkable state of affairs, illustrated in table 1, does more than prevent a simple treatment of the precipitin reaction according to the law of mass action, for it also prevents characterization of this and other immune reactions by adsorption isotherms, as has been attempted from time to time, for adsorption isotherms also contain a concentration term.

Another difficulty in the quantitative formulation of the reaction was due to the realization that the anticarbohydrate in Type III antipneumococcus horse sera was not a single substance,

but a mixture of antibodies of greatly differing reactivities. This was clearly shown (23) by the presence of residual, difficultly precipitable antibody after the serial addition of small quantities of S III, the occurrence of a portion of antibody precipitable at 0° but not at 37°, and the precipitability of only a part of the antibody by S III which had been methylated.

TABLE 1\*

*Effect of volume and final concentration of antibody N precipitated*

VOLUME	ANTIBODY B 62 AT 0°C.		ANTIBODY B 61 AT 37°C.	
	Antibody N pptd. by 0.03 mgm. S III	Final concentration antibody N	Antibody N pptd. by 0.05 mgm. S III	Final concentration antibody N
ml.	mgm.	mgm. per ml.	mgm.	mgm. per ml.
2	0.87	0.21		
4	0.91	0.10	0.87	0.25
6	0.87	0.07		
8	0.84	0.06	0.87	0.12
10	0.84	0.05		
12	0.87	0.04	0.85	0.08

\* Adapted from J. Exp. Med., 1935, 61, 563.

#### QUANTITATIVE FORMULATION OF THE S III-ANTIBODY SYSTEM

With the aid of several assumptions, however, it was found possible to derive from the law of mass action a relation which accounts quantitatively for the S III-antibody reaction (23) and many other instances of the precipitin reaction as well. These assumptions and simplifications are:

1. S III and antibody (A) are chemically and immunologically multivalent with respect to each other; that is, each substance possesses two or more groupings capable of reacting with the other.

2. Although the anticarbohydrate is known to be a mixture of antibodies of different reactivities it may be treated mathematically as if its average behavior were that of a single substance, A.

3. For convenience of calculation the S III-antibody reaction is considered as a series of successive bimolecular reactions which take place before precipitation occurs.

4. The mass law applies, so that the rates of formation of the reaction products are proportional to the concentrations of the reacting substances.

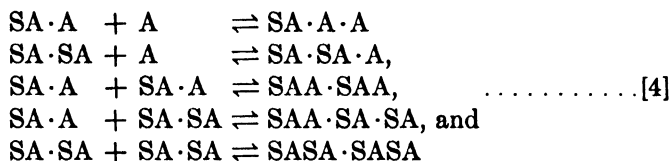
The reactions postulated are, in arbitrary units:



followed, for example in the region of excess antibody, by the competing bimolecular reactions due to the mutual multivalence of the components:



A third step would follow, in which the competing bimolecular reactions would be:



in which the first two reactions would occur only in the presence of enough A to carry the composition of the reaction product beyond the  $SA_2$  stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances, and the reaction would continue until particles would be formed large enough to settle from the solution. Precipitation would take place at this point, doubtless facilitated by the mutual discharge, with loss of affinity for water, of ionized or polar groupings brought together by the series of chemical reactions (cf. also 4).

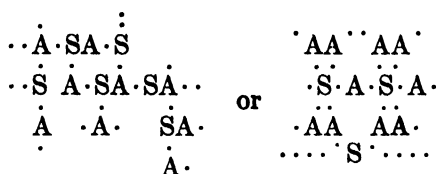
If A and S III are mixed in equivalent proportions the SA formed in reaction [2] would merely polymerize in steps [3], [4] . . . , and the equivalence-point precipitate would be  $(SA)_n$ .

In the region of excess S III similar expressions would apply, in which S and A would be interchanged in [3], [4], . . . . In the presence of a large excess of S, in the inhibition zone, there would also be present in solution a soluble compound,  $S_xA$ , containing



one more molecule of S in combination than the last insoluble compound (18). Since this is formed only with a very large excess of S, all of the specific groupings of A would tend to react with S rather than with SA complexes and there would be no large, insoluble intermolecular aggregates formed.

The final precipitate, then, would in each case consist of antibody molecules held together in three dimensions by S III molecules,



a view similar to that put forward also by Marrack (4) but, it is believed, more definite and more easily treated quantitatively. The process of aggregation as well as the initial hapten-antibody combination is considered to be a chemical reaction between definite molecular groupings.

Since no evidence of dissociation could be found over a large part of the reaction range, the equilibria postulated evidently lie very far to the right. The mathematical treatment of these reactions has been given elsewhere (23). In applying the derived equations to experimental data it is necessary to convert units of S and A into milligrams. This is accomplished by defining one unit of antibody N as 1 milligram. A may then be put equal to the number of milligrams of antibody N precipitated at the equivalence point (midpoint of the equivalence zone) and R equal to the ratio of A to milligrams of S III precipitated (added) at the same reference point. The equation most frequently applicable then becomes

$$\text{mgm. of antibody N precipitated} = 2RS - \frac{R^2 S^2}{A} \dots\dots [5]$$

The theoretical significance of a and b in the empirical equation [1] is now clear, for  $a = 2R$  and  $b = \frac{R^2}{A}$ . Both of these constants have the immunological and chemical significance given above.

If, instead of the difficultly determinable "equivalence point" the reference point for R and A be taken as either end of the equivalence zone, depending on the precipitin system studied, it will usually be found possible to avoid the complicated  $R - 3R$  equations also given in (23) and use only the simpler  $R - 2R$  equation [5]. Table IX in (23) shows the agreement between found and calculated values to be very close for a number of antibody solutions and sera. In the use of equation [5] it is assumed that all of the antibody is precipitated at the reference point. If this is at the beginning of the equivalence zone, as in the S III - A system, the assumption is not entirely correct, for the amount of A precipitated does not reach a maximum experimentally until S is present in appreciable excess. For the complete description of the behavior of a serum in the precipitin reaction a separate determination of the maximum amount of specifically precipitable nitrogen is necessary.

If both sides of equation [5] be divided by S, the resulting equation,

$$\frac{\text{mgm. antibody N precipitated}}{\text{mgm. S precipitated}} = 2R - \frac{R^2}{A} S \dots\dots\dots [6]$$

is that of a straight line. This linear relationship makes it possible to characterize an unknown Type III antipneumococcus serum or antibody solution in the region of excess antibody by two or, better, three analyses, in duplicate. If the ratio of antibody N to S III precipitated be determined for two or three different amounts of S III in the region of excess antibody and a straight line be drawn through the points so obtained, the intercept on the y axis =  $2R$  and the slope =  $-\frac{R^2}{A}$ . With the R and A values at

the beginning of the equivalence zone calculated in this way the amount of antibody nitrogen precipitated by any quantity of S III less than  $\frac{A}{R}$  may be calculated with a fair degree of accuracy.

(For the linear relation, also, see Fig. 1.)

In the region of excess S III the behavior of a serum as far as the beginning of the inhibition zone may be characterized by the determination of the A and S III precipitated at two points,

since in this region the terms of equation [6] may be inverted and the linear relation

$$\frac{S \text{ pptd.}}{A} = 2R' - \frac{(R')^2 A}{\text{Total } S}$$

applies if  $R'$  be taken as the  $\frac{S}{A}$  ratio at the end of the equivalence zone at which S III appears in excess,<sup>\*</sup>  $A$  be taken as the amount of antibody N precipitated, and  $\frac{S \text{ pptd.}}{A}$  be plotted against  $\frac{1}{\text{Total } S}$ .

In the inhibition zone, in which large quantities of S III are present and the amount of precipitate has begun to diminish, this equation is no longer applicable and it is necessary to determine the apparent dissociation constant of the soluble compound  $S_xA$  (cf. also (26)).

Despite the wide variation in the behavior of individual sera the above expressions permit the complete description of the precipitin reaction between S III and an unknown antiserum without an unduly burdensome number of microanalyses or the sacrifice of a large amount of material.

In all of the experiments described above horse sera or antibody solutions obtained from horse sera were used, but equations of the same form, with constants of smaller magnitude, were found to hold as well for antipneumococcus Type III sera produced in rabbits (27).

#### PRECIPITATION IN PROTEIN-ANTIPROTEIN SYSTEMS

Extension of these studies to protein-antiprotein systems was more complicated, since it was necessary to distinguish between antigen nitrogen and antibody nitrogen if the composition of the

<sup>\*</sup> In (23) p. 590, "total S" in this equation was taken as the (constant) amount of S combined with A precipitated at this reference point, while A in the equation was made to vary by defining it as that portion of the constant amount of antibody precipitated throughout this zone given by the fraction  $\frac{\text{"total S"}}{S \text{ added}}$ . Owing to space limitations this explanation was unfortunately omitted.

precipitate was to be directly determined. This was accomplished by the use of a red protein dye, R-salt-azo-biphenyl-azo-crystalline egg albumin, which was freed from fractions reactive in most anti-egg albumin sera and then injected into rabbits (26, 28, 29). In the specific precipitates produced by the dye and antibody, antigen was estimated colorimetrically after solution of the washed precipitate in alkali. The entire solution was then rinsed quantitatively into a micro-Kjeldahl flask for a total nitrogen determination, after which the amount of antibody nitrogen could be calculated by deducting from the total N the amount of dye-antigen N found colorimetrically. Equations [5] and [6] were applicable in this system as well, also an empirical equation,

$$\text{mgm. antibody N pptd.} = 3RD - 2\sqrt{\frac{R^2 D^2}{A}} \dots\dots [7]$$

in which R = the A:D ratio at the maximum for antibody N precipitated, D = the amount of dye N precipitated, and A = the maximum precipitable antibody N. This equation permitted calculation of the maximum specifically precipitable nitrogen with avoidance of a separate set of analyses for the determination of this constant. In this system the composition of the precipitate could be estimated by direct analysis over the entire reaction range, and the ratios of the components were found to vary without discontinuity from higher to lower A: dye values as relatively larger amounts of dye were added to the antisera. This was also shown by the increasing redness of the precipitates. Azoprotein-antibody systems were also studied by Marrack and Smith (20b) and by Haurowitz and Breinl (30). Both groups confirmed the varying composition of the precipitate, the latter workers having previously reached similar conclusions regarding hemoglobin-antibody precipitates (31).

With the aid of the information gained from the precipitin reaction between R-salt-azo-biphenyl-azo-crystalline egg albumin and its homologous antibody it was found possible to study a colorless protein, crystalline egg albumin, and its homologous antibodies. This instance of the precipitin reaction was also

found to be quantitatively described over a large part of the reaction range by the theory, and the equations were applied to unorganized analytical data accumulated by other workers (32). The crystalline horse serum albumin (33) and mammalian thyroglobulin systems also behaved in accordance with the theory (34). The precipitin reactions of antipneumococcus sera other than Type III were likewise found to be described by the same equations derived from the theory (27, 35), and these relations were also found by Pennell and Huddleson (36) to cover the reactions of anti-*Brucella* goat sera with the appropriate antigens.

With hog thyroglobulin and antisera produced in rabbits (34) it was possible to test directly an assumption made with S III, crystalline egg albumin, and crystalline serum albumin. It had been postulated that in the region of excess antibody and in the equivalence zone all of the presumably pure hapten or antigen added was precipitated if the exceedingly sensitive precipitin test on addition of more antibody to a portion of the supernatant failed to reveal the presence of antigen. This assumption had been criticized by Taylor, Adair, and Adair in the course of their studies on the egg albumin system (37). A direct test with egg albumin did not seem easy to devise, but with highly purified thyroglobulin (38) it was found that 96 to 101 per cent of the iodine added was precipitated in the region of excess antibody. These observations and the quantitative data recorded (34) also render untenable the view subsequently advanced by Clutton, Harington, and Yuill (39) that thyroglobulin is not an antigen.

The quantitative precipitin technique affords an exceedingly accurate method for the estimation of minute quantities of specific polysaccharides (21) and of small quantities of proteins (32). Determination of the amount of specific nitrogen precipitated in the region of antibody excess from a previously calibrated antiserum permits the quantity of antigen in the sample to be read off from the specific nitrogen (antigen N + antibody N) calibration curve. The amount of nitrogen actually measured is usually many times that due to the antigen, so that very small quantities of antigen may be accurately determined. This method has been applied to the estimation of albumin and globu-

lin in body fluids by Goettsch and Kendall (39A), and has been used as a guide for the isolation and identification of protein fractions from normal and pathological human sera by Kendall (39B).

#### SPECIFIC BACTERIAL AGGLUTINATION

At the inception of the present work there was available for the study of the mechanism of bacterial agglutination no quantitative method conforming to the criteria of analytical chemistry. It was found that the microanalytical technique used in the case of the precipitin reaction could be modified so as to permit the estimation of agglutinins for pneumococcus with a high degree of accuracy. A measured amount of thoroughly washed, killed pneumococcus M (S) (40-2) or S (R) (43) suspension was analyzed for nitrogen. This value was deducted from the nitrogen found after the same volume of cell suspension had been agglutinated by an accurately measured volume of serum and washed free from non-specific protein. The difference gave, in milligrams per milliliter, the amount of agglutinin nitrogen removed by the bacterial cells under the conditions used, and, when the proportions of pneumococci and serum were chosen so as to leave the cells in excess, gave the agglutinin content of the serum in absolute, not relative, terms. As in the precipitin reaction, the amount of agglutinin found was shown to be independent of the non-specific protein present, and to depend on the relative proportions in which the components were mixed and not on the final concentration of antibody. Though Type I pneumococcus and homologous antibody reacted according to the same type of equation as in the precipitin reaction, the agglutinin reaction was actually found to be simpler, since the exigencies imposed by the reactive bacterial surfaces limited the range of combining proportions of bacterial polysaccharide and antibody (42). As might have been anticipated from the view that specific bacterial agglutination differs from specific precipitation only in that the former reaction takes place on particulate matter, the latter between two dissolved reagents (44), type-specific pneumococcal antiscarbohydrate was found to be quantitatively the same

whether measured as agglutinin or as precipitin. It was also found that the entire course of these instances of specific bacterial agglutination could be accounted for, as in the precipitin reaction, on the basis of a chemical reaction between multivalent antibody and multivalent antigen, without assumptions as to electrical potential or cohesive force such as those made by Northrop and de Kruif (45).

The quantitative methods introduced were found applicable in several instances of cross reactions as well, and yielded data showing the wide variations in the reaction course when compared with the corresponding homologous reactions (29, 36). The cross reactions between Types III and VIII pneumococci, their type-specific polysaccharides, and Types VIII and III antipneumococcus sera were shown to involve a relatively small proportion of the total antibody, especially in sera produced in the rabbit (46). This was taken to indicate that the immunological (chemical) unit responsible for the specificity of each of the two polysaccharides involved was larger than the simple glucuronic acid or aldobionic acid portion common to both, as had been maintained up to that time (47), and this view has now been accepted (48).

#### SOME CONSEQUENCES OF THE QUANTITATIVE THEORY

The quantitative theory of the precipitin and agglutinin reactions discussed above was proposed by its authors, in avowed realization of many of its weaknesses, as a makeshift and a first attempt at a general quantitative theory of two important immune reactions. On this basis it has not only explained much that is not accounted for by the older, essentially qualitative theories, but has also served as a working hypothesis that permitted several rather far-reaching predictions which might not otherwise have been foreseen.

In the first place the method for the estimation of the maximum specifically precipitable nitrogen, the absolute measure of the precipitin content of a serum, was the outgrowth of the initial oversimplification in which direct application of the mass law was attempted (18). The earlier oversimplification did not survive (cf. 20, 23), but the method based upon it is a standard one to-day.

In the second place, a study of the effect of strong salts on the reaction between pneumococcal polysaccharide and homologous antibodies (49) showed that the lessened precipitation and decrease (table 2) in the values of both constants in Equation [5] was not due to increased solubility of the precipitate. On the basis of the quantitative theory (23) a reversible shift in the equilibrium between polysaccharide and antibody was indicated,

TABLE 2

*Effect of the concentration of sodium chloride upon the reaction between S III and antibody*

FINAL NaCl CONCENTRA- TION	HORSE ANTIBODY SOLUTION B 36					RABBIT ANTIBODY SOLUTION B 50*	
	0.1 M	0.15 M	0.51 M	0.93 M	1.79 M	0.15 M	0.93-0.98 M
S III used	Nitrogen precipitated						
mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
0.02	0.54	0.50	0.42	0.39	0.36		
0.05	1.13	1.03	0.90	0.84	0.75	0.43	0.24
0.075	1.41	1.41	1.29	1.15	1.03	0.60	
0.10	1.75	1.66	1.54	1.28	1.22	0.77	0.34
0.15	1.78	1.86	1.62	1.50	1.45	1.04	0.39
0.20†	1.82	1.85	1.70	1.58	1.51	1.18	0.41
Equations; mgm. antibody N pptd...	27.5 S-	25 S-	22.2 S-	20.2 S-	18.1 S-	9.5 S-	5.0 S-
	104 S <sup>2</sup>	84 S <sup>2</sup>	72 S <sup>2</sup>	68 S <sup>2</sup>	57 S <sup>2</sup>	18 S <sup>2</sup>	15 S <sup>2</sup>
A†.....	1.82	1.86	1.71	1.50	1.44	1.25	0.42

\* Prepared according to Felton, J. Immunol., 1931, **21**, 357.

† Excess S III.

‡ Calculated mgm. antibody N pptd. at antibody-excess end of equivalence zone. (Reprinted from J. Exp. Med., 1936, **63**, 819.)

and it was predicted that this shift would permit the isolation of pure antibody. For example, 0.1 mgm. of S III precipitated 1.24 mgm. of antibody N from a given serum in physiological saline (0.15 M NaCl), but formed an insoluble compound with only 1.01 mgm. of antibody N in 1.75 M NaCl. On the assumption that the equilibria involved were reversible, it was considered possible that if the reaction were first carried out in 0.15 M



NaCl, the non-specific protein were then washed out, and 1.75 *M* NaCl were next added, 0.23 mgm. of N should be dissociated in the form of pure antibody (49). Actually, antibody solutions of 90 to 98 per cent purity

$$\frac{\text{specific precipitin N} + \text{agglutinin N}}{\text{Total N}}$$

were readily attainable in this way in a single step from many unrefined antipneumococcus horse and rabbit sera of various types (50). With improvements in technique analytically pure antibody globulin was isolated (51, 52) through the use of the method. Studies on the purified antibodies led to the discovery that pneumococcal anticarbohydrate produced in the horse had a high molecular weight, while the same antibody (also anti-egg albumin) produced in the rabbit was of the size of the principal component of normal globulin. This phase of the work, which has been reviewed elsewhere (7), and accompanying quantitative studies (27) furnished much of the theoretical background (cf. also (53)) and practical methods of control for the use of antipneumococcus rabbit sera (53) and antibodies (54) in the treatment of pneumonia.

Verification of another prediction, made from the quantitative agglutinin theory, puts the function of salts in this immune reaction on a basis different from the currently held view. The reversibility of the precipitin reaction, in the sense that a precipitate may be shifted from one region of the reaction range to another by addition of antigen or antibody, warranted the assumption of a similar reversibility for the closely related agglutinin reaction. For example, Type I pneumococci may be agglutinated with a large excess of antibody, and the excess of antibody then removed by thorough washing and the agglutinated pneumococci resuspended evenly in saline. According to the theory, the prediction may be made that addition of an appropriate amount of Type I pneumococci or of Type I specific polysaccharide will cause reagglutination into larger clumps. This would be brought about through the chemical linkage of multivalent antibody on the agglutinated, washed cells either with multivalent S I on the

freshly added pneumococci, or with dissolved S I if a solution of the polysaccharide were added instead. It may also be predicted that if Type II pneumococci or Type II polysaccharide be added to a similar suspension of agglutinated, washed, Type I cells, reagglutination into larger clumps will not occur, although salt concentration, electrical potential, and cohesive force (45) would be identical, or nearly so, in the two sets of experiments. It may also be predicted that addition of Type I pneumococci *after* the Type II cells added in the preceding instance will result in reagglutination, leaving a turbid supernatant containing most of the Type II cells. All of these predictions are fully verified when subjected to experimental test (42), and the verification is interpreted in the light of the above theory as follows:

Specific bacterial agglutination is not a mere combination or coating of bacterial surface antigen with dissolved antibody, followed by non-specific flocculation due to the presence of salts, but appears more reasonably to be a more dynamic process: the chemical combination of multivalent antigen on the reactive bacterial surfaces with multivalent antibody, originally in solution, to build up larger and larger aggregates until these flock out and the process is terminated. The function of salts in this process is then the purely secondary one of minimizing electrostatic effects due to the presence of many ionized groupings on the particles, effects which might interfere with the primary process of building up aggregates by chemical interaction. At least in the case of the water-insoluble antibody to pneumococcus produced in the horse, salts also provide ions for the soluble, ionized salt complexes in which form this antibody probably reacts.

Even though the initial bimolecular antigen-antibody reaction on the bacterial surface may take place in the absence of electrolyte, the reactants carry ionized groups and it is evident that the succeeding competing bimolecular interactions between polysaccharide molecules on partly sensitized cells and additional antibody in solution or on other cells would soon result in the formation of particles carrying large numbers of ionized groups. Coulomb forces on such particles, in the absence of electrolyte,

are known to cause abnormally great viscosities and Donnan effects, so that it would not be surprising if these forces would prevent the continuation of the chemical reactions resulting in the completion of what is commonly recognized as specific bacterial agglutination. Only when the effect of these forces is reduced by a sufficient ionic atmosphere, on addition of electrolyte, is it possible to obtain significant figures for viscosity, osmotic pressure, sedimentation constants, and the like. To ascribe a similar rôle to electrolytes in specific bacterial agglutination would seem reasonable and consistent, for after reduction of the Coulomb forces the growing particles could again interact chemically, and the process of agglutination be completed. An analogous explanation of the function of salts in the reaction between heterogenetic antigen and antibody had already been given by Brunius (55) but this was not known until after publication of (42).

It will be noted that the effect ascribed to salts is essentially the same as in the older hypothesis. However, recognition of this effect as dependent upon the building up of aggregates between multivalent antigen and multivalent antibody simplifies the problem, abolishes the uncertainties and inconsistencies of the older view, and permits the precise definition of the conditions for specific bacterial agglutination. Whether or not a given antigen-antibody mixture will agglutinate, and which components, if any, will not be carried down when the (specific) aggregation takes place may now be predicted on the basis of the theory. Moreover, the prediction may be made even though the potential and cohesive forces in agglutinating and non-agglutinating systems are essentially similar.

In the above reagglutination experiments it may be considered that in the initial agglutination of relatively few pneumococci with relatively much antibody the dynamic process of combination of multivalent bacterial antigen with multivalent antibody has been interrupted at an early stage. In accord with this are the small size of the clumps formed and the ease with which the agglutinated cells may be resuspended, just as in precipitin reactions carried out with an excess of antibody the specific

precipitate may be relatively easily homogenized and resuspended. In the experiments under discussion the dynamic agglutination process was then continued under controlled conditions. It was found that resumption of agglutination of the Type I pneumococci could occur only when the chemical reaction of multivalent S I with multivalent antibody could go to completion, and that introduction of a chemically unrelated antigen such as Type II pneumococci or S II produced no effect, even though the potential had been suitably lowered by the presence of salt. These experiments are in entire agreement with the

TABLE 3\*

*Molecular composition of specific precipitates from rabbit antisera*

ANTIGEN	EMPIRICAL COMPOSITION OF SPECIFIC PRECIPITATE				COMPOSITION OF SOLUBLE COMPS. IN INHIBITION ZONE
	At extreme antibody excess	At antibody excess end of equivalence zone	At antigen excess end of equivalence zone	In inhibition zone	
Ea	EaA <sub>1</sub>	EaA <sub>1</sub>	Ea <sub>2</sub> A <sub>1</sub>	→ EaA <sub>2</sub> →	(EaA)
DEa	(DEaA <sub>1</sub> )	(DEaA <sub>1</sub> )	DEa <sub>2</sub> A <sub>1</sub>	→ DEa <sub>4</sub> A <sub>1</sub>	DEa <sub>2</sub> A ?
Sa	SaA <sub>1</sub>	SaA <sub>1</sub>	SaA <sub>1</sub>	→ SaA <sub>2</sub> →	(SaA)
Tg	TgA <sub>10</sub>	TgA <sub>14</sub>	TgA <sub>10</sub>	→ TgA <sub>2</sub> →	(TgA)
S III	SA	S <sub>1</sub> A <sub>1</sub>	S <sub>1</sub> A	→ S <sub>4</sub> A	S <sub>1</sub> A

Ea = cryst. egg albumin (32); DEa = dye egg albumin (26); Sa = cryst. serum albumin (33); Tg = thyroglobulin (34); S III = pneumococcus, Type III. specific polysaccharide (27).

A = Antibody, S = Minimum polysaccharide chain weight reacting. Data in parentheses are somewhat uncertain.

\* Reprinted from the Journal of the American Chemical Society, 1938, 60, 242.

conception of specific bacterial agglutination given above and also support Topley, Wilson, and Duncan's experiments (56) leading to the same conclusions.

If these conclusions are valid it is possible that the so often cited analogies between specific immune aggregation and the aggregation of suspensions in general have been misleading in their emphasis on a supposedly non-specific phase in the process. It is possible, also, that the knowledge gained in the quantitative chemical study of these immune reactions will be of service in clarifying the behavior of other systems of colloidal suspensions,

in which the chemical reactions involved in aggregation are far less well defined and understood. If the tables were turned in this way it would not be without its elements of humor.

Another outcome of these quantitative and theoretical studies has been the possibility of calculating, for the first time, the actual molecular composition of the specific precipitate at the principal reference points and in the principal zones of the entire precipitin reaction range. Marrack and Smith had calculated that at the flocculation optimum one molecule of pseudoglobulin antigen combined with about four molecules of antibody (20a), but use of more recent data on the molecular weights of antibodies (58, 59) has permitted the assignment of empirical formulas (table 3) over much of the reaction range in a number of systems (60). While these formulas cannot be considered as those of definite chemical compounds conforming to all criteria of homogeneity they represent faithfully at least the empirical composition of the specific precipitate at the reference points chosen and, in general, lie within such limits as to justify the application of classical chemical treatment to the study of the precipitin reaction.

#### MOLECULAR FORMULAS FOR ANTIGEN-ANTIBODY COMPOUNDS

With the use of the formulas in table 3 as a basis, a two-dimensional and therefore necessarily incomplete graphic representation of the entire reaction range between crystalline egg albumin (Ea) and antibody (A) might be offered (fig. 2). In this scheme the possibility of the combination of Ea with six molecules of A is taken to indicate that ordinarily up to six molecular groupings, not necessarily all different, in the Ea molecule may react with A; in other words that Ea has six immunological (chemical) valences, or a multiple of six. For simplicity the assumption is also made that the average A molecule has two valences or combining sites for Ea, but the possibility of additional bonds is not excluded.

The egg albumin studies (32, 60) have also shown that the immunological "valence" of the Ea depends to some extent on the breadth of reactivity of the antibody, and that this, in turn, generally varies with the length of the immunization period to

which the animal furnishing the antibody is subjected. If the minimum immunological "valence," or number of combining groups, of the antibody entering into specific precipitation is 2, it is probable that this increases during the course of immunization as the antibody becomes capable of reacting with more and more groupings on the antigen molecule. This is merely the expression, in chemical terms, of the well-known overlapping of specificities on prolonged immunization. Many antisera also contain antibody which behaves as if it possessed only a single immunologically reactive grouping, since it does not precipitate

*Compounds in the Region of Excess Antibody*

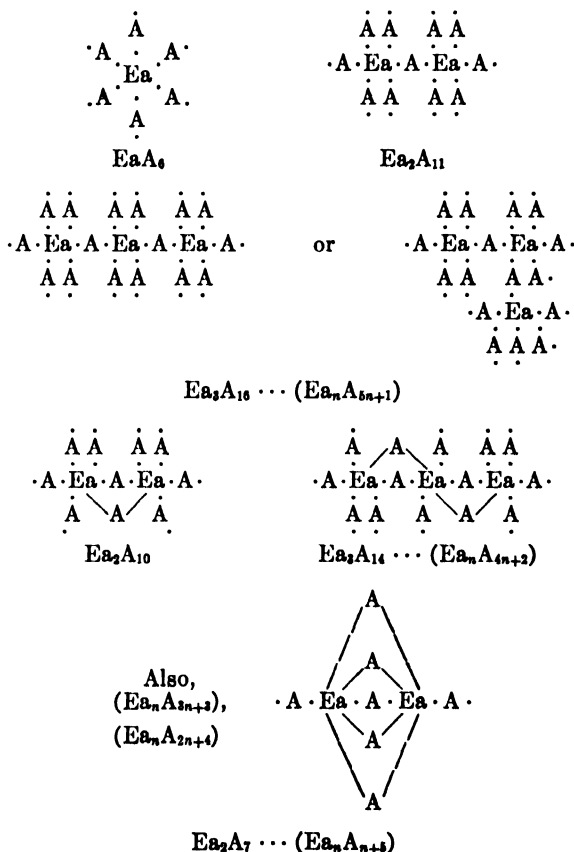


FIG. 2. MOLECULAR FORMULAS OF EGG ALBUMIN-ANTIBODY COMPOUNDS INDICATED BY ANALYSES AND THE QUANTITATIVE THEORY



and more antibody in the antisera from successive courses of injections is required to precipitate a given amount of Ea. Pre-

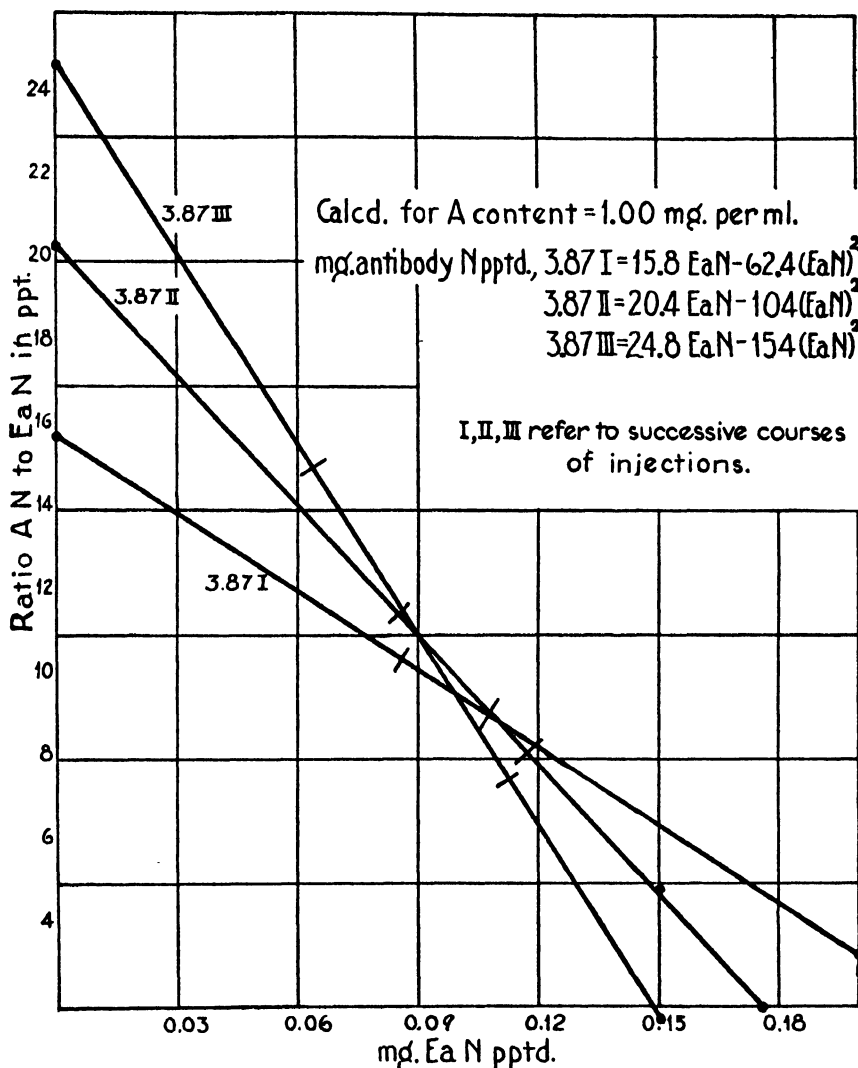


FIG. 3. Adapted from J. Exp. Med., 1935, 62, 697

sumably for the same reason, that is, the probable ability of the antibody to react with more and more groupings in the Ea molecule as immunization proceeds, this tendency is reversed in the



region of excess antigen. In this zone the antibody becomes more and more efficient with continued immunization in that a given amount of antibody combines with more and more antigen. It is precisely this region which is of the greatest interest in serum therapy, for passive immunization of an infected patient is achieved through the region of antigen excess. A series of charts similar to figure 3 could also be constructed from the pneumococcal polysaccharide-antibody data in (27).

Extension of the molecular weight studies (58) to egg albumin-antibody precipitates dissolved in an excess of Ea revealed the presence of several dissolved substances of higher sedimentation constant than those of either Ea or A, affording evidence of the presence in this system, as well, of relatively simple, soluble Ea-A compounds in the inhibition zone. If, on the other hand, the specific precipitate had been merely "peptized" by the excess of Ea there would be no reason why the process should stop at discrete molecular entities larger than either of the components. According to recent calculations of Tiselius and Kabat made from electrophoresis diagrams (61) these inhibition zone compounds are probably  $Ea_2A_3$  and  $(Ea_2A_3)_2$ , with higher polymers also present if the solutions are not allowed to stand long enough to come to equilibrium.

These experiments not only throw light on the mechanism by which specific precipitates are dissolved or prevented from forming by excess of antigen, but even furnish a possible clue to the vexing question of why inhibition does not likewise occur at the antibody excess end of the precipitin reaction range. This has been ascribed by Marrack to the close-packing of antibody around the antigen in the region of antibody excess, with consequent loss of polar groups and decrease in solubility, while at the antigen excess end the antigen, with more combining sites than antibody, would not permit close-packing (4). The present calculations tend to support and extend this view, for it has been shown that at the antibody excess end the composition of the precipitate in several systems is of the same order as for the Ea system, namely,  $EaA_4$  to  $EaA_6$  (60) (See also table 3). This would involve the close association of relatively many A molecules, especially if

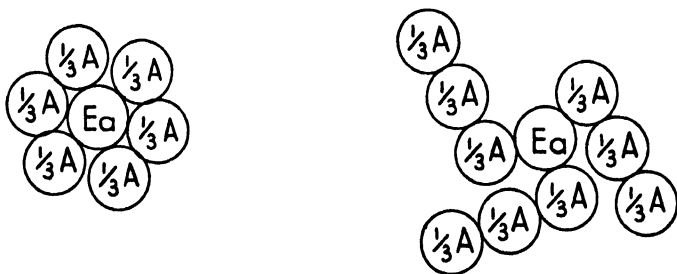
polymerization occurred at the same time in some such way as indicated in figure 2. At the other end of the system, where relatively small, simple, soluble compounds have been shown experimentally to be present (58, 61), a more extended configuration of such molecules can be pictured (figure 2).

#### ANALYSIS OF OBJECTIONS TO THE QUANTITATIVE THEORY

From the above discussion it will be clear that the conception of specific precipitation and agglutination as the combination of multivalent antigen with multivalent antibody entails as a consequence both the qualitative and quantitative explanation of much that has hitherto been obscure. However, the mere agreement of even large bodies of data with a theory does not guarantee its validity, nor does its serviceability as a tool exclude the possibility that an alternative theory might serve even better. Objections to the qualitative as well as quantitative features of the theory have been made, and these will now be considered.

A sizable portion of the evidence that antigens may possess multiple determinant groups has been supplied by Hooker and Boyd (62). In a discussion leading to the conclusion that the aggregation phase of specific precipitation is non-specific (63) these workers maintain that the assumption of multivalence or multiple determinant groups in the antibody molecule is unnecessary and complicating. However, both antigens and antibodies as proteins possess the same basic structure, and immunological as well as chemical multivalence follows naturally from what is known of this structure. Moreover, an antibody may function as an antigen (cf. Landsteiner and Prasek (64) Ando (65), Marrack and Duff (66)) and in this capacity its multivalence would necessarily be conceded. Why, then, need a multivalent antigen be a univalent antibody? Hooker (67) has, moreover, argued for the necessity of subdividing a combining group on the antibody into three parts to account for specificity differences between *dextro*- and *levo*- isomers. This would seem to imply a limited form of multivalence, for each part would presumably be a chemical bond. Also, in discussing the question

of valence Hooker and Boyd admit (63) the "tacit" assumption of multivalence in calculations relating the antibody: antigen ratios at the equivalence point to the molecular weight of the antigen. But the assumption appears to the writer more than "tacit," and if Hooker and Boyd now believe antibody to be univalent they must abandon the entire basis for these and others of their calculations. For purposes of calculation antibody molecules were assumed by Boyd and Hooker (68) to consist of three (four would perhaps have been better) spherical Svedberg units of 34,500. At the equivalence point (a point definable only as a mean value in an often broad equivalence zone) it was assumed that "the antigen molecule is just completely covered by molecules of antibody" in such a way that "each of the three component units is in contact with the antigenic surface." This, of course, implies at least one combining group on each Svedberg unit, otherwise there would be no reason for the supposedly flexibly joined units to attach themselves to the antigen surface. But it is well known that antigen can combine with at least twice as much antibody as at the "equivalence point." It must then either be assumed that the additional antibody combines with that already on the antigen (and there is no evidence than any part of the antibody is more loosely combined than the rest) or it must be assumed that antibody may also combine head on,<sup>4</sup> so to speak. The two modes of combination might be indicated two-dimensionally as follows for the Ea-A system:



<sup>4</sup> Combination of this type, also, is now proposed by Boyd and Hooker (*J. Gen. Physiol.* 1939: 22, 281).

The latter alternative is merely another type of diagram such as that given in figure 2, for the A valences remaining free could, and doubtless would, combine with other Ea-A combinations. It also follows from this that the calculation (63, footnote pp. 343, 344) that 15 antibody molecules would more than cover the surface of an antigen the size of the hemocyanin of *C. irroratus* cannot be correct, so that the deduction from this calculation must also be abandoned.

Aside from the valence inconsistency the above hypothesis fails to account for the existence of the whole series of equivalence-zone compounds between EaA<sub>3</sub> and EaA<sub>2</sub>, which are often experimentally demonstrable and which are easily accounted for on the basis of multivalent antigen and antibody (cf. also fig. 2). Hooker and Boyd themselves appear to have receded from a too rigid application of their theory of the relation between the molecular weight of the antigen and its combining ratio with antibody at the "equivalence point" (cf. 32, p. 718; also Ref. 57).

The argument for the multivalence of antibody is further strengthened by evidence for the existence of univalent antibody as well. In the papers on the quantitative theory it has been shown that in addition to antibodies which are considered multivalent there is also antibody which behaves as if it were univalent, since when isolated it can no longer combine with antigen to form aggregates, but can only do so when more complete, or multivalent, antibody is present (32). If flocculation is merely a non-specific consequence of antigen-antibody combination due to the presence of salts such antibody should not exist. Its existence, however, has been repeatedly demonstrated, and it has also been shown to combine with antigen (unpublished experiments of the writer).

In support of the non-specific aggregation of the antigen-antibody complex Hooker and Boyd have submitted experiments on the mixed agglutination of red cells. It will be recalled that Abramson (69) had shown that mixtures of red cells and bacteria with antisera to both agglutinated to form mixed clumps and not separate, homogeneous aggregates as would seem to be demanded by the chemical theory, and as was indeed actually shown to

take place by Topley, Wilson, and Duncan (56) in the case of two different bacteria and their antisera. A legitimate criticism of Abramson's experiment would seem to be that the relatively enormous size of the red cells could have blocked the free movement of agglutinating bacteria and so prevented homogeneous agglutination of each cell species. It is also quite possible that the large size of red cells accounts for the mixed agglutination observed by Hooker and Boyd.

Very recently Boyd and Hooker (70) have reported that red cells were agglutinated in the presence of a huge excess of antibody, when presumably every reactive site on the surface was occupied by a molecule of antibody and there appeared to be no opportunity for the formation of aggregates through free antigen linkages. This is taken to indicate non-specific aggregation for this set of conditions. This would, however, be only a limiting case for the theory that aggregation is merely a continuation of the process of combination of multivalent antigen and multivalent antibody, just as the solution of the precipitate, or absence of precipitation in the region of great antigen excess is also a limiting case. As already noted on pp. 71-5, in both instances relatively simple molecules are formed. In the antigen-excess region structural considerations and the molecular ratios of the components favor soluble compounds. In the antibody-excess region symmetry and close-packing (4) favor insolubility. An insoluble *molecule* of EaA<sub>6</sub>, for example, would probably combine with similar molecules to build up aggregates through forces which might be termed non-specific, although possibly due in part to the combination of suitable polar groups on colliding EaA<sub>6</sub> molecules to form salt-like linkages much the same as those in any inorganic precipitate. The texture of precipitates produced in this way, is, however, generally very fine, and differs from the form of those in which relatively less antibody is used and antigen valences are left free to combine with bound as well as unbound antibody. Over this, the principal range of the precipitin and bacterial agglutination reactions, the aggregates become larger and larger and more gelatinous as the chemical reactions involving union of multivalent antigen with multivalent antibody

take place in more nearly equivalent proportions, or, in Mar-rack's terminology, as lattice formation becomes more and more complete. At the other (antigen) end of the range lattice formation also fails, but this is adequately accounted for on the basis of the union of multivalent antigen with multivalent antibody, as already explained. Boyd and Hooker appear willing to concede lattice formation (with univalent antibody?) in the region of antigen excess.

As matters now stand, there is evidence that so-called non-specific factors may determine flocculation or agglutination in the special case in which all antigen valences are occupied by antibody. Over by far the greater part of the reaction range, however, with the exceptions indicated in the first and last diagrams of figure 2 the possibility remains that chemical linkages of partly "coated" antigen molecules occur through antibody molecules, and much has been explained on this basis that cannot be accounted for on the older theory. It would seem reasonable to conclude that such chemical linkages would occur with greater "avidity" when structural and kinetic considerations show them to be possible than would the more vague non-specific linkages.

Hooker and Boyd have also studied the rate of flocculation in the precipitin reaction, and have found that in the region of antibody excess there is a linear relation between the antigen dilution and the time of flocculation (71). Under the assumption that in this range the antigen particles are maximally coated with antibody a simplified form of v. Smoluchovski's equation for slow colloidal flocculation was considered applicable since it resulted in a linear relation. However, inspection of the table (p. 374) shows that much of the linear range in several of the antigen-antibody systems considered lies in the region in which the composition of the precipitate is known to change with the proportions of the components, and hence the antigen cannot be maximally "coated." There is therefore every reason for the belief that  $\epsilon$  in v. Smoluchovski's theory would be variable, and not constant, as assumed, so that the data do not permit application of the theory except over a much more restricted range. If the linear relation applies, nevertheless, in the region of varying

composition, it is probable that this is due to a balancing of some of the many unknown factors influencing the rate of flocculation. The experiments quoted therefore offer evidence of non-specific flocculation only in the limiting range covered in the work quoted in the preceding paragraph, and this evidence is weakened by the apparent validity of the linear relation in reaction regions in which it cannot have the theoretical significance given.

The increased speed of flocculation demonstrated by these workers in mixtures is less easy to explain away. As admitted by Hooker and Boyd, there was cross-reactivity of related antigens in one-half of the experiments cited in (63) and these must therefore be excluded. But cross-reactivity could not account for the increased speed of flocculation in the other instances, and this was predictable on the basis of non-specific flocculation. A similar effect has recently been observed by Duncan (72) in mixed Ea-serum albumin-antibody systems, but the flocculation rate of mixed agglutinating systems was not increased. Duncan concluded from this that only chemical aggregation is involved in specific bacterial agglutination, but that, in addition, non-specific factors intervene in specific precipitation. Because of the far-reaching analogies between these two immune reactions, this view seems unlikely and would seem equivalent to merely another way of saying that there are still unknown factors which influence the rate of specific precipitation.

Hooker and Boyd also state (63) that the reagglutination experiment discussed in connection with the quantitative theory (pp. 66-70) may be explained by a dissociation of antibody from the initially agglutinated cells, instead of by a reagglutination of the entire mass due to the combination of multivalent S on the freshly added cells with multivalent antibody on the initially agglutinated cells. If by "dissociation" they mean antibody soaked off by the saline in which the initially agglutinated cells were suspended, this was excluded by the conditions of the experiment, for the cells were repeatedly washed until the supernatants no longer agglutinated added homologous pneumococci. If, on the other hand, it is meant that the added cells carry to completion an exceedingly slight dissociation due to any revers-

ibility of reactions [2], [3], etc., the following evidence to the contrary is available: As in the precipitin reaction, the composition of the agglutinated cells is independent of the antibody concentration at equilibrium, so that any change of this kind due to dissociation and reversibility is too slow to measure. Moreover, if there were a redistribution of antibody to include the newly added cells the aggregates formed should be smaller than before, whereas microscopic and macroscopic observations show them to be strikingly larger, as would be expected if the S on the added cells combined with A on agglutinated cells to link masses together.

Although Eagle (73) has accepted the multivalence of antigen and antibody<sup>5</sup> he has raised objections to the view that flocculation is a consequence of such union (74). However, there was no difficulty in interpreting the action of formaldehyde on antibodies, cited by Eagle, in the light of the chemical theory of flocculation, and as this has been discussed fully in a recent publication (35) repetition would seem unnecessary.

Deductions regarding the mechanism of the precipitin reaction have been made by Haurowitz (75, 76) as a result of his studies on arsanilic acid azoproteins and their reaction with antibodies. Many of these deductions seem questionable since they rest on the assumption that the antigens used were well-defined, single substances. The evidence against this, even in Haurowitz's work, is very strong, for in serial additions to excess antisera only 60 per cent and 33 per cent of the antigens added were actually found in the first, and largest, precipitates in two experiments ((76), Tables II and III, pp. 396, 397). The objections raised by Haurowitz against the quantitative theory (76) seem to rest on a misunderstanding, except that the variable composition of the precipitate is ascribed exclusively to the presence of a number of different antibodies. The views expressed regarding the forces responsible for antigen-antibody combination are based on the Breinl-Haurowitz theory of antibody formation (31), modified to take account of the possible ionic nature of at least the initial

<sup>5</sup> In (35) this statement was inadvertently so worded that the acceptance of aggregation as a consequence might also have seemed implied.



combination (18) and additional influences of polar groups indicated by the work of Chow and Goebel (77). Since these matters are also discussed in detail in (4b) they will not be further touched upon here.

Criticism of the quantitative theory has also been made by Marrack (4b, pp. 470, 471). The occurrence of irreversible bimolecular reactions is doubted in systems which we and others have repeatedly shown to be reversible in the sense that the composition of the precipitate may be changed by addition of either component or by alteration of the salt concentration. The inconsistency involved is admitted, but a high degree of irreversibility must be assumed to exist under a given set of conditions, otherwise the effect of concentration of the component in excess would be greater. The real difficulty probably lies in the assumption that antibody may be treated as a single substance, when, as we and others have frequently shown, it is a mixture of antibodies of different reactivities. We have repeatedly called attention to this oversimplification of the quantitative theory as it now stands, but it at least permits many calculations and predictions to be made with accuracy and a certain degree of utility. As already stated (23, 26, 32), the theory was offered, in the realization of many weaknesses, as a temporary expedient which might be useful until antibody possessed of uniform reactivity could be isolated.

With regard to Marrack's other objection regarding the order in which the various bimolecular steps were considered to take place, the same answer may be given; namely, that it was realized that the reaction did not take place in steps, but that for purposes of calculation it was convenient to make such arbitrary subdivision. If some such scheme be envisaged as that in Fig. 2, and the reactions are assumed to involve individual linkages or valences, the reaction order used for the arbitrary "units" originally adopted does not seem so improbable.<sup>6</sup>

It has also been stated by Malkiel and Boyd (57) that the equations of the quantitative theory do not apply in the region

<sup>6</sup> Progress in computations along this line has already been made and should be reported shortly (private communication from Dr. F. E. Kendall).

of antigen excess in the hemocyanin-antibody system. Equation [5] is cited, also the equation

$$\text{mgm. antigen precipitated} = 2R'A - \frac{(R')^2 A^2}{\text{antigen added}} \dots\dots [8]$$

(equivalent to [5] with S and A transposed), which is said not to apply in the zone of partial inhibition. When these equations were proposed (23) it was expressly stated that they did not apply in these zones. Malkiel and Boyd therefore emphasize what they evidently consider a weakness of the quantitative theory. The writer, however, is inclined to consider as advantageous the division of the precipitin reaction into definite zones which can be delimited experimentally, especially if this permits the application of a theory and consequent calculations and predictions which do not follow from any purely empirical relation. Moreover Malkiel and Boyd also state that equation [8] and the relation derived from it,

$$\frac{\text{antigen pptd.}}{\text{antibody N pptd.}} = 2R' - \frac{(R')A}{\text{antigen added}} \dots\dots\dots [9]$$

which is linear with respect to  $\frac{1}{\text{antigen added}}$ , do not apply to the hemocyanin system, but insufficient data are given to permit a test of this assertion. The necessary figures were most kindly sent to us and equation [8] (hence [9]) was found to fit for the entire region of maximum antibody precipitation in three out of the four instances tested. We have, then, for this portion of the reaction range, our own equation, derived from the law of mass action, and the empirical relation of Malkiel and Boyd. As for the inhibition zone, many of the figures in the tables given by these workers (57) clearly indicate that the linear relation can be extended into the inhibition zone only with a sharp inflection horizontally. Thus in Table II p. 387, R in the two inhibition-zone precipitates is patently constant for Serum 926<sub>2</sub>; R is also constant, within the large experimental error, for the three inhibition-zone precipitates of Serum 928, Table V, p. 380, and for the three precipitates in this zone from Serum 928<sub>2</sub>, p. 381.

In three of the six instances cited, then, the data do not warrant the extension of Malkiel and Boyd's empirical relation into the second zone to which it is said to apply. In two other antigen-antibody systems for which our own data are sufficiently accurate, two out of four sera studied have shown constant composition of the precipitate in the zone of partial inhibition ((23), p. 567; (32), p. 235). It may be pointed out that precipitates of constant composition in equilibrium with the soluble inhibition-zone compound are entirely consistent with the theory of the union of multivalent antigen with multivalent antibody.<sup>7</sup>

While the above detailed discussion of the precipitin reaction and specific bacterial agglutination may have seemed overlong, these two immune reactions are the only ones for which a considerable body of precise, absolute data exists. Since this review concerns itself primarily with such data these two reactions have necessarily taken up most of the space allotted.

#### THE TOXIN-ANTITOXIN REACTION

A beginning has been made, however, toward placing another of the most important immune reactions on a similar basis. Until recently the voluminous knowledge of the toxin-antitoxin reaction could be expressed only in relative terms such as were

<sup>7</sup> We have had the privilege of discussing this matter with Mr. Malkiel and Dr. Boyd and have sent them this paragraph for further comment, which is appended herewith.

Rejoinder by Mr. Malkiel and Dr. Boyd: We realized that [5] was not intended to apply to the region of antigen excess, nor [8] to the inhibition zone, but did wish to emphasize what still seems to us a weakness in the theory, which accounts for the precipitin reaction quantitatively only by dividing it into three regions, with a different equation for each. We regret not including enough auxiliary data to enable the reader to try [8]. We meant to say [8] did not apply in the inhibition zone ("large antigen excess"), and pointed out this was evident from its form, without numerical test. To us, the zone of antigen excess and the inhibition zone seem continuous and essentially similar. We feel that the fit obtained with some of our data and [9] is simply that always possible if a curvilinear relation is tested against linear data for a relatively small portion of its mathematical range; our experimental errors are admittedly relatively large. If our empirical relation is really a straight line, it is impossible that [9], which, instead of  $R$  (our symbols), contains  $1/R$ , should also be straight, if plotted similarly.

formerly used for the precipitin and agglutinin reactions, and for the same reasons. With the isolation of what is presumably pure diphtheria toxin by Eaton (78) and by Pappenheimer and Johnson (79) the antigen has become known as a protein, the properties of which may be followed quantitatively. In the flocculation zone of the reaction the methods of analysis developed for the quantitative study of the precipitin and agglutinin reactions have also been found applicable.

The first study of this nature was made by Marrack and Smith (80), who showed that diphtheria toxin-antitoxin floccules were mainly "denatured" pseudoglobulin, and that the amount of nitrogen precipitated was independent of the quantity of non-specific serum proteins present and increased with increasing amounts of antitoxin (A) up to the flocculation limit. In sera showing an *in vivo*: *in vitro* ratio of 1 or greater, Healey and Pinfield (81) found that if the composition of the toxin-antitoxin floccules were represented as TA (in units) at the Ramon flocculation point, the composition  $TA_2$  could be attained when A was present in twice the amount. Over all but the ends of the flocculation range all of the T and A present were in the precipitate. It was also found that TA floccules could combine with A or a relatively small amount of T, and that  $TA_2$  floccules combined with T, but with very little A. The reversibility of the Danysz effect (cf. also (82, 83) was demonstrated.

A more detailed quantitative study was made by Pappenheimer and Robinson (83), using highly purified toxin, and in some instances purified antitoxin. It was found that the flocculation zone corresponded to the equivalence zone of precipitin reactions in which neither component is demonstrable in the supernatants, in accord with Healey and Pinfield. The nitrogen figures cited indicate approximately a three-fold range of combining proportions over the entire zone. Since the Danysz effect is shown outside the zone of flocculation, where any effect due to the combination of T and A in varying proportions should be immediately reversible, it is postulated that T and A combine rapidly to form a soluble compound, followed by a slow reaction resulting in flocculation when the proportions are suitable. The initial

rapid reaction would be responsible for the Danysz effect and this would be slowly reversible after addition of the last portion(s) of T.

The amount of N per Lf unit of T was calculated by subtracting the N precipitated from 300 units of A by 200 Lf of T from that precipitated by 400 units. In six sets of determinations the values ranged from 0.00042 to 0.00048 mgm. of N, with a mean of 0.00046, regardless of the purity of the T or A used, and agreeing with the value given by Eaton (78) for his purest T. With this value it is shown that a constant figure is obtained for the A precipitable throughout the equivalence zone except with A showing evidences of alteration. The value found was, in general, 0.016 mg. of N per flocculation unit of A. Using these figures, the A:T (or more strictly, A N:T N) ratios at the A-end, flocculation point, and T-end of the equivalence zone were found to be approximately 7.0, 3.5, and 2.5, respectively. With the aid of the flocculation-point ratio and a single pair of duplicate nitrogen determinations the potency of both an unknown toxin and an unknown antitoxin may be calculated, even if no standard is available for comparison!

#### THE RÔLE OF LIPIDS

Quantitative analytical methods both for antibody and lipids have been used by Horsfall and Goodner (84) in studying the relation of lipids to specific precipitation and agglutination in antipneumococcus sera. Of the many significant observations and analyses made only those will be discussed bearing directly on the mechanism of the precipitin reaction. It was found that the first precipitate formed carried down a relatively high proportion of the lipid present, and lipid contents as high as 51 per cent were noted. Subsequent precipitates in the same serum were relatively low in lipid and the amount in any case showed no relation to the nitrogen (protein) content. Thorough extraction of the lipids abolished specific precipitation and agglutination in the case of horse serum, and greatly diminished these effects in rabbit serum, in line with the earlier work of Hartley (85),

but lecithin restored these properties to extracted horse serum and cephalin to extracted rabbit serum. It was considered that most of the lipid carried down was adsorbed to the specific precipitate, but that the exceedingly small amount of lecithin or cephalin necessary to restore the precipitating and agglutinating power might indicate that antibody consisted of a lipo-protein complex. Highly purified antibody from horse serum was also stated to contain lecithin (84d). It was, however, found that the extracted antibody could still combine with pneumococci, although these were not agglutinated, so that an alternative hypothesis seems to the writer at least equally probable. The observation, quoted above, that the first precipitate in a series contains relatively much lipid, and that subsequent precipitates contain less lipid (and often form more slowly) might be taken to indicate that the function of lipid in promoting specific precipitation and agglutination is essentially a mechanical one. The effect of the lipid would then be to provide nuclei for the formation of aggregates, much as dust particles in a supersaturated solution of a substance promote its crystallization.

In the foregoing review immune reactions involving specific precipitation and bacterial agglutination have been discussed in the light of newer data obtained by quantitative methods conforming to the criteria of analytical chemistry. An outline has been given of the progress made, since the introduction of these methods, toward the understanding of these immune reactions, and instances have been given of the utility of the methods and the theories based on them. The analytical methods have withstood all tests for accuracy and reliability and are now quite generally accepted as standard, but the quantitative theory, in spite of its utility, is obviously defective in certain respects. It is hoped that this discussion of the present state of these problems will stimulate not only the search for more rigorous theoretical explanations than are now available, but will also further the application of suitable absolute methods to other, more intricate manifestations of immunity.

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# THE PATHOGENIC STAPHYLOCOCCI

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During the past several years the pathogenic staphylococci have received considerable attention, particularly with regard to their toxigenic capacities and the relation of their toxins to staphylococcal infections. Papers between 1924 and 1931 by Parker (201, 258), Gross (102-110), and Burnet (26-28), and by many others in the years immediately following, have yielded new information, have established a new basis for the interpretation of known facts about staphylococci, and occasionally have reëmphasized earlier observations upon staphylococci.

Burnet's study of these organisms followed an incident in Bundaberg, Queensland, in 1928, which demonstrated the toxi-



genic potentiality of staphylococci. Of twenty-one children who received a routine prophylactic injection of diphtheria toxin-antitoxin, twelve died within 34 hours with acute staphylococcal toxemia (144). A culture of the toxin-antitoxin yielded *Staphylococcus aureus*.

A brief review of some of the newer information was made by Holman (126) in 1935. Considerable material has continued to appear, and it has seemed advisable to summarize some of the contributions to the study of staphylococci that have been made during the past decade or so. Such a summary must necessarily be limited in scope in this review; the immunology and therapy of staphylococcal infections must be omitted. A limitation is also imposed by the very newness of some of the material, which will acquire significance only through the perspective to be gained by the solution of present problems. However, it is to be hoped that a summary at the present time will serve to emphasize certain salient features of recent investigations, and will afford points of departure for future work.

#### EXOTOXIN

Definite demonstrations of the production of toxins by staphylococci were made by the earlier investigators (159, 190, 195, 229, 251). These reports received only passing attention, however, and little emphasis was subsequently given to the toxigenic capacities of staphylococci, or the possible rôle of toxins in the pathogenesis of staphylococcal infections. From the more recent information it is now firmly established that certain strains of staphylococci produce a soluble exotoxin, which may be obtained in culture filtrates, and which is quite comparable to other recognized bacterial toxins in its antigenicity and other properties.

In accordance with the effects produced by potent culture filtrates, various self-descriptive terms have been employed, *viz.*, lethal toxin, dermonecrotxin (or dermatoxin), hemotoxin, leucocidin, enterotoxin, and coagulase and fibrinolysin. This variety of effects raises the question whether some of them may be due to a single toxin whose action varies with the cells with

which it comes in contact, or whether each toxic effect is due to a separate toxic factor. Regardless of the ultimate solution of this question, the terms at present supply a convenient nomenclature in the discussion of the activity of pathogenic staphylococci.

Under the heading "exotoxin" is to be described the soluble antigenic toxic substance produced by many pathogenic staphylococci. Toxic filtrates of staphylococcal cultures produce death (from lethal toxin) or dermonecrosis (from dermonecrotxin) when injected into experimental animals in suitable doses and by the proper routes. Since hemolysis of rabbit erythrocytes is considered by many as another manifestation of the action of exotoxin (26, 28, 86, 115, 192, 217-223), a discussion of "hemotoxin" may for convenience be included under the general heading of exotoxin, subject to such reservations as will be indicated below.

Exotoxin in varying amounts appears to be produced by many, but by no means all, pathogenic staphylococci. From 85 to 90 per cent of strains studied by Dolman (60) and by Stookey and Scarpellino (237) were reported to produce demonstrable amounts of toxin. Our own unpublished findings are quite comparable to these. However, it appears that a much smaller proportion of strains produces a potent lethal toxin (24, 192, 229).

#### *Preparation of staphylococcal toxin*

In the study of staphylococci attention has necessarily been paid to the determination of the optimum conditions for the formation of their toxins. In many laboratories at present culture media are used that are based ultimately upon those described by Walbum (255) and by Parker (201). Essentially the method now in common use is to grow staphylococci in broth or on a semisolid medium (containing less than one per cent agar) with a veal- or beef-infusion base, in an atmosphere of 20 to 40 per cent CO<sub>2</sub>, for one to three days, or occasionally longer. Modifications which have been adopted in various laboratories generally involve slight alterations in the amounts of various ingredients, the reaction of the medium, and methods

of harvesting and preserving the toxins. The composition of the medium appears to be of considerable importance, and its modification may be a source of confusion in the interpretation of results (25, 207).

Valuable information has been obtained concerning the nutritive requirements of staphylococci. Following the demonstration by Hughes (130) and by Knight (153) of growth-accessory factors, subsequent work has shown that nicotinic acid or nicotinamide and thiamin (vitamin B<sub>1</sub>), effective in minute amounts, are required by the staphylococcus for its growth (71, 118, 125, 154, 155, 158, 161). The requirement for these factors is highly specific, for even closely related compounds are ineffective in promoting growth (155, 156, 161). Richardson (216) has shown that uracil is necessary for the anaerobic growth of staphylococci, its requirement also being highly specific.

It is apparent that the use of synthetic media for the production of toxins would be desirable, for it would allow a more accurate study of the nature of staphylococcal toxin than has yet been possible. Staphylococci will grow satisfactorily in an entirely synthetic medium containing the growth accessory factors (71), and recently Gladstone (89) has been able to obtain good toxin in a medium of known chemical composition. Media of somewhat simplified composition have been employed for the successful production of the toxin. Leonard and Holm (163) have described a "semi-synthetic" medium, which is used in an atmosphere of 80 per cent CO<sub>2</sub>. Media prepared with the dialysate of nutrient broth have been used by Holt (127) and by McClean (188). These possess the advantage of containing distinctly less nitrogenous material than the whole broth, and the toxins obtained appear to be antigenically equal to those obtained by the methods more generally employed.

The introduction of the use of semisolid agar (27) and of an atmosphere of partial CO<sub>2</sub> tension (203) represent distinct additions to the methods of preparation of staphylococcal toxin. Birch-Hirschfeld (8) obtained potent toxin by growing staphylococci on the surface of cellophane on agar plates. She attributed this to autolysis of the cocci by metabolic products which did

not diffuse through the cellophane. It has been shown by McClean (188) that the action of cellophane, of agar, and of some other materials, is to adsorb from the medium a substance, as yet unidentified, which inhibits toxin-formation.

While the presence of CO<sub>2</sub> may directly stimulate growth of the cocci, as it is known to do in the case of certain other bacteria, its chief advantage appears to be its action in maintaining the reaction of the medium at a level which best favors the formation of toxin. Bigger (6) reported that CO<sub>2</sub> is definitely prejudicial to the formation of toxin by an occasional strain of staphylococcus. To prevent excessive alkalization, Bigger adopted a combination of glycerol and a phosphate buffer. Apparently a similar effect was obtained by Nélis, Bouckaert, and Picard (192), who reported that 0.1 per cent of glucose favored toxin-production.

The possible association of oxidation-reduction processes with the production of staphylococcal toxin is suggested in recent work by McBroom (185). She has noted that the production of hemotoxin by staphylococci is definitely correlated with their ability to reduce methylene blue. No such correlation was found with their proteolytic, lipolytic, or carbohydrate-utilizing capacities. Burky (21) has reported that a toxigenic strain of staphylococcus when grown anaerobically produced lethal toxin, but no hemotoxin.

#### *Properties of staphylococcal exotoxin*

Like some other bacterial toxins, staphylococcal exotoxin is readily thermolabile. Loss of toxicity is generally reported to occur between 55° and 60°C., but no reference is made to the effect on antigenicity (57, 158, 258). The toxin is sensitive to light and oxidation. Li (166, 167) has reported the complete destruction of the toxic properties (lethal, necrotic, hemolytic) by the action of light and methylene blue, with no loss of antigenicity.

The concentration and purification of toxin was attempted by Burnet and Freeman (28), who used the methods often applied to other bacterial toxins. By precipitation with acetic acid a

yield of from 60 to 90 per cent was obtained, much of the nitrogenous matter of the crude toxin being removed. Precipitation with trichloroacetic acid was successfully employed by Boivin and Izard (14). Reference has been made above to the use of a dialysate medium by Holt and by McClean for the production of toxin free from the non-dialyzable nitrogenous material of the whole broth.

Ultracentrifugation of staphylococcal hemotoxin by Gratia and Nélis (101) resulted in a fluid the upper layers of which contained the toxin, and the lower layers none. There was, however, no appreciable concentration of the toxic factor.

Although exotoxin is filtrable, much of it may be lost by adsorption on certain types of filter (229, 262). Woolpert and Dack found that lethal toxin, dermonecrotin, and hemotoxin were retained to a great extent by Seitz filters, while Berkefeld "N" candles yielded filtrates of high potency (262). This is comparable to similar findings for staphylococcal leucocidin (248) and for staphylocoagulase (72).

Nélis, Bouckaert, and Picard (192) reported that in equal concentrations mineral acids destroy the toxin, while organic acids vary in their action, from partial destruction to none at all. The speed of the reaction had a direct relation to the temperature. Colloidal iron and manganese have been reported to inhibit lethal toxin, while colloidal gold, silver, and platinum had no effect (162). Neither of these reports refers to the effect of these agents upon antigenicity.

Llewellyn Smith (169) has recently reported that glycerol, ethylene glycol, sucrose, and glucose inhibit the dermonecrotic action, due to partial destruction of the toxin. Experiments to determine the effect on antigenicity were inconclusive. The lethal toxicity for rabbits was also significantly diminished, but the hemolytic action was unaltered. In the latter instance this was probably due to the lower concentration of glycerol used in hemolytic tests, for in equal concentrations glycerol inhibited hemolysis and dermonecrosis in parallel fashion. The effect of glycerol should be remembered when the dermonecrotic test

is used to titrate antitoxic sera that have been preserved with glycerol.

Rigdon and his associates (4, 218, 221) found that hemotoxin and dermonecrotin were inhibited by hypertonic solutions of sodium chloride, and by certain other salts of sodium, potassium, and magnesium. This was attributed to some action of the salts on the cells which rendered them refractory to the action of the toxin. Lithium chloride also inhibited dermonecrosis but had no effect on hemolysis. Weinstein (257) reported that lecithin inhibited hemolysis in blood agar by staphylococci and streptococci, and that cholesterol exerted an antagonistic action, preventing the inhibition by lecithin. The lethal, necrotic, and hemolytic capacities of toxin were reported by Schwartz (230) to be considerably reduced by olive oil.

#### *Staphylococcal Toxoid (Anatoxin)*

The effect of a suitable concentration of formaldehyde is well known, since it is the accepted agent for detoxifying toxin in the preparation of toxoid. In a concentration of 0.3 to 0.5 per cent complete detoxification is accomplished at 37°C. in from 7 to 20 days. The lethal, dermonecrotic, and hemolytic properties are destroyed, without loss of the antigenicity. In concentrations up to one per cent, more rapid detoxification, accompanied by greater antigenicity, has been reported by Kitching and Farrell (151). Burnet and Freeman (29) found that the rate of detoxification bears a direct relation to the concentration of hydroxyl ions and possesses a high temperature coefficient.

The immunizing power may be enhanced by precipitation of toxoid with alum (69, 163), with saturated ammonium sulfate (127), or with trichloroacetic acid at pH 4.0 (209). The alum-toxoid is particularly effective as a primary stimulus in immunization (69).

Tests for antigenicity of toxoid involve the determination of binding and flocculating properties, and animal inoculations. The biological standardization offers certain difficulties, which have been discussed by Llewellyn Smith (168). Factors which

are involved include dosage and time intervals, great individual variation in response to the antigen, the effect of diet and seasonal variations, and differences in response to various types of antigen, such as toxoid, alum-toxoid, and toxin-antitoxin or toxoid-antitoxin floccules. However, Dolman and Kitching (62) regard the biologic method as of only qualitative value for toxoids of low immunizing efficacy. Kitching and Farrell (151) have reported a rough correlation of binding power and the response *in vivo* to antigenic toxoid preparations.

#### *Antigenicity of staphylococcal toxin and toxoid*

The possibility of producing antitoxin in high titer by a suitable course of immunization was demonstrated in laboratory animals by Parker (201), Burnet (26), Dolman (57), and Gross (109), in horses by Parker and Banzhaf (202) and by Burnet (28), and in human volunteers by Dolman (57). In reports supplying repeated confirmation of this, the experimental production of complete antitoxic immunity has sometimes been claimed, and the protection thus established was frequently related to an increased titer of circulating antitoxin. A basis was thus established for the therapeutic use of toxoid and antitoxin.

Conflicting reports of the therapeutic value of staphylococcal toxoid and antitoxin are found in the literature. While certain types of staphylococcal infection appear definitely to benefit from this type of therapy, some unfavorable results may be attributed to uncritical selection of cases, and also to a premature conception of the part played by toxin in the pathogenesis of certain staphylococcal infections.

The serologic problems raised have necessitated a common basis for the comparison of results. Under the direction of the Committee on Standards of the League of Nations an international standard staphylococcal antitoxin has been established (115). Antisera are titrated for their ability to inhibit hemolysis, dermonecrosis, or the lethal action of toxin.

#### THE ACTION OF STAPHYLOCOCCAL EXOTOXIN

Laboratory animals are susceptible in varying degrees to the lethal effects of the toxin. The rabbit is particularly susceptible,

and usually is the animal of choice. Wild rabbits used by Kellaway, Burnet, and Williams (143) were reported to be five times more susceptible than the domestic varieties. Guinea pigs are definitely less susceptible than rabbits, as is shown by the relatively larger effective dose required for lethal or dermonecrotic tests (27, 60, 162). Adult animals, both rabbits and guinea pigs, appear to be relatively more susceptible than young animals (23, 57, 162), although young animals have sometimes been used (262). Cats, dogs, monkeys, mice, rats, horses, and doves show varying susceptibility (57, 60, 143, 217, 229, 262). The toxin is administered intravascularly. There appears to be good evidence that when given by mouth, exotoxin is without effect (60).

In considering the lethal potency of staphylococcal toxin, it must be remembered that the toxins studied to date generally represent relatively crude filtrates of cultures. Consequently the figures for the minimal lethal dose do not approach the order of those reported for some other toxigenic bacteria. The M.L.D. of staphylococcal toxin, following injection into the blood stream of rabbits, has generally been reported as between 0.1 and 0.5 ml. per kilogram of body weight (21, 26, 57, 60, 143, 163, 199, 262). Roy has recently recorded lethal titers ranging down to 0.0016 ml. per kilogram (228).

It has been well established that the ability of a strain of staphylococcus to produce exotoxin is in no way related to the severity of the infection from which it was isolated (57, 192, 198, 200, 232). A similar lack of relationship was reported by Minett for strains from veterinary sources (182).

#### *The lethal action of toxic filtrates*

A striking series of events follows the intravenous injection of a potent staphylococcal toxin into a susceptible animal (26, 57, 60, 143, 195, 217, 229, 262). The reaction may be immediate (death resulting in five to fifteen minutes), or it may be delayed (death occurring in two to four hours, and more often in twenty-four hours or longer). For a brief interval the animal appears to be normal. Then, in rapid sequence, it becomes unsteady and paralysis of the hind legs develops; the respiration, which may



be rapid at first, becomes irregular and gasping; incoördinate running movements often occur; initial contraction of the pupils is followed by wide dilatation. The animal then usually dies after violent convulsions, or it may become moribund until death supervenes. There is often incontinence of urine and feces, and sometimes diarrhea.

When death is delayed for a few hours, a somewhat similar series of events occurs, but more slowly. Opisthotonos, and a marked loss of weight (sometimes as much as 200 grams per day) have been recorded by Burky (21) in rabbits surviving for more than twenty-four hours. In cats, vomiting was described in the earlier stages by Kellaway, Burnet, and Williams (143). It is significant that the symptoms exhibited by the children who died of staphylococcal toxemia at Bundaberg were quite similar to the experimentally produced reactions described above.

Investigating the mode of death, Kellaway, Burnet, and Williams showed that in addition to a direct action on the heart (an effect sometimes preventable by active or passive immunization), the toxin also affects the vascular supply of the lungs (143). This results in obstruction of the pulmonary circulation, with consequent acute failure of the right heart. An initial transient fall of blood pressure occurs, apparently due to pharmacologically active constituents of the culture medium. This is followed by recovery to or above normal with maintenance of the blood pressure at about the normal level. An increased output of adrenalin aids this recovery (52, 143). Finally there is a characteristically rapid terminal fall of blood pressure, occasioned by failure of the pulmonary circulation (143).

The possibility has been considered that histamine in the toxin might be responsible for the characteristic rapid death of experimental animals (26, 57, 219); but this has been dismissed by Burnet (26) and by Dolman (57). More recently Feldberg and Keogh (70) have reported that histamine is liberated during perfusion of the guinea pig's and cat's lung with toxin. They suggested that this might explain the acute fall of arterial pressure, rise in pulmonary pressure and peripheral vasodilatation.

Several more or less detailed studies have been made of the pathologic changes which follow the intravenous administration

of exotoxin (16, 52, 90, 92, 143, 192, 217, 219, 225, 226). The chief features that are seen in the gross and confirmed microscopically are serous or serosanguinous exudate into the pericardial, pleural, and peritoneal cavities, congestion of various organs, and a characteristic finding of hemorrhages, petechial or extensive, in the organs and serous linings of cavities. The majority of blood vessels are dilated, and intravascular hemolysis, which may be quite intense, is frequently found (143, 192, 261). Injury to the tissues is indicated by changes which vary from cloudy swelling to intense necrosis.

*The dermonecrotic action of toxic filtrates*

The necrotic action of staphylococcal toxin is exemplified by the reaction of dermonecrosis. This was carefully studied and described by Parker (201), and has received repeated confirmation by others. Dermonecrotxin appears to parallel the lethal toxin in its production, properties, and antigenicity. The reaction appears frequently to serve as well as the lethal action in determining the toxic effect of potent filtrates. The general description of exotoxin given above applies essentially also to dermonecrotxin, and requires no further elaboration at this point.

*The hemolytic action of toxic filtrates*

In addition to their lethal and necrotic properties, many toxic staphylococcal filtrates are also capable of hemolyzing erythrocytes. Evidence obtained from tests for hemolysin and neutralization of hemolysin by antiserum suggested the existence of more than one hemolysin (or hemotoxin). Glenny and Stevens (91) have demonstrated that there are at least two antigenically distinct hemotoxins, which they have designated as " $\alpha$ -toxin" and " $\beta$ -toxin." This has received ample confirmation by Bryce and Rountree (19) and others. The commonly described staphylococcal hemolysin is  $\alpha$ -hemotoxin, active at 37°C. on both rabbit and sheep erythrocytes, and frequently characteristic of pathogenic staphylococci from human sources. The  $\beta$ -hemotoxin is inactive on rabbit cells, but is active against sheep cells, and then only after incubation at 37°C., followed by incubation at

a lower temperature. It does not appear to be related to human pathogenicity. Although produced by some strains of human origin,  $\beta$ -hemotoxin appears to be quite characteristic of veterinary staphylococci (19, 182). These strains usually produce both  $\alpha$ - and  $\beta$ -toxins, but some strains produce  $\beta$ -toxin almost or entirely to the exclusion of  $\alpha$ -toxin.

Morgan and Graydon (183) have demonstrated the existence of two  $\alpha$ -hemotoxins, which they call " $\alpha_1$ -toxin" and " $\alpha_2$ -toxin." The latter was usually present in only small amounts in a toxic filtrate. Flaum and Forssman (76) noted the distinction between lysins for rabbit and sheep cells, and suggested that  $\alpha$ -toxin was a mixture of two lysins. It is possible that one of these corresponds to the  $\alpha_2$ -toxin of Morgan and Graydon.

The  $\beta$ -hemotoxin appears to correspond to the "hot-cold" lysin studied by Bigger (6) and Bigger, Boland, and O'Meara (7), and previously reported by Walbum (254). Roy (228) and Flaum (75) have suggested that the lysin active on human erythrocytes is identical with  $\beta$ -toxin, but Roy's human lysin also hemolyzes rabbit erythrocytes.

The physical properties and antigenicity of  $\alpha$ -hemotoxin are quite similar to those of exotoxin. In contrast, the  $\beta$ -hemotoxin appears to be thermostable (6, 7, 19, 263), although Minett (182) reported little difference in resistance to a temperature of 55°C. An antigenic toxoid was prepared by Bryce and Rountree (19) by the action of formaldehyde on  $\beta$ -toxin. Levine (164, 165) has suggested that the combination of red blood-cells and lysin may be due to adsorption, for the reaction was found to conform to the general principles controlling adsorption phenomena.

The susceptibility of the erythrocytes of various animal species to  $\alpha$ -hemotoxin is of interest, and considering the variations in technique among different laboratories, the broad general correspondence is striking. The following table lists in decreasing order the sensitivity of various mammalian erythrocytes to  $\alpha$ -hemotoxin:

Bryce and Rountree (19): Rabbit, Ox, Koala, Sheep, Ferret, Rat, Human, Guinea pig.

Dolman (57): Rabbit, Sheep, Cow, Guinea pig, Human, Cat, Horse.

Forssman (77): Rabbit, Ox, Sheep, Goat, Human, Horse,<sup>1</sup> Guinea pig.<sup>1</sup>

Gross (107): Rabbit, Cow, Sheep, Goat, Human, Horse, Guinea pig.

Le Fèvre de Arric (162): Rabbit, Guinea pig.

Minett (182): Rabbit, Ox, Sheep, Dog, Human.

Woolpert and Dack (262): Rabbit, Mouse, Dog, Monkey, Rat, Sheep, Guinea pig, Human.

Regarding susceptibility to  $\beta$ -toxin, bovine (19, 182) and human (75, 228) red cells have been reported to rank close to sheep cells. In comparison to sheep cells, Bryce and Rountree recorded the susceptibility to  $\beta$ -toxin of the red cells of several other species in the following approximate decreasing order: ox, human, koala, ferret, guinea pig, rabbit, and rat. Except for the ox cells, which were about as sensitive as sheep cells, all of these showed little or no hemolysis. Minett (182) found that the erythrocytes of the dog and horse were relatively resistant to  $\beta$ -toxin.

It would appear that hemolysis in blood agar should not be considered as any more than suggestive of the production of hemotoxin, and certainly the use of blood-agar plates does not lend itself to a quantitative interpretation. It is well known that hemolysis in blood-agar is not necessarily related to the production of soluble hemotoxin (243, 244). Hallman (114), in this laboratory, has reported that 91.4 per cent of 480 strains of staphylococci from nasal mucous membranes hemolyzed human blood-agar. Only 67 per cent of these strains were potential pathogens. Comparable results were obtained recently by McFarlan (189).

#### *Correlation of the toxic factors*

The belief has been expressed by a number of authors that lethal toxin, dermonecrotxin, and  $\alpha$ -hemotoxin are probably identical (26, 28, 86, 115, 192, 217-223). Claim for the unity of the toxins was based upon similarity of physical properties, and upon the fact that all three were found in quantitative relationship in the filtrates studied, and were quantitatively neutralized by a single antiserum. When quantitative differences

<sup>1</sup> No hemolysis.

between the toxic factors of certain filtrates occurred, they were attributed to the destruction or attenuation of one or more of the toxic functions of these filtrates, possibly in the "toxophore" group of antigenically identical antigens (26, 29).

On the basis of adsorption experiments with erythrocytes the identity of hemotoxin with lethal or dermonecrotxin has both been claimed (86, 192) and denied (258). Adsorption tests performed by Roy (228) were inconclusive, but suggested a relationship between hemotoxin for sheep and human cells, and a difference between this lysin and that active on rabbit cells. The results of all of these experiments were rarely clear-cut, and at best are only suggestive of the relationships claimed.

Gengou (86) has suggested that, rather than the constant presence of the three toxins in filtrates to indicate unity, the absence of one of them from given filtrates would be better proof of their lack of identity. This condition appears to have been realized in some of the filtrates described by Parker (201), Burky (21, 24), and Flaum and Forssman (76). Parker reported that many of her "non-poison-producing strains" (which produced no dermonecrotxin) were strongly hemolytic, and that filtrates containing potent dermonecrotxin were usually ineffective when injected intravenously into rabbits. Flaum and Forssman studied antigenic hemolytic filtrates which were neither lethal nor dermonecrotic. Burky reported the production of lethal toxin, but no hemotoxin, anaerobically in hormone broth and aerobically in Uschinsky's medium. Studying a strain which originally produced both lethal and hemolytic toxins, he found that after two years' maintenance of the stock strain on artificial media, its ability to hemolyze had almost completely disappeared, and coincidentally its toxicity had increased. The implications of the results of Parker, of Flaum and Forssman, and of Burky should receive serious consideration.

#### LEUCOCIDIN

It is of historical interest that the earliest description of a bacterial leucocidin was that of the staphylococcus, being described in 1894 by Van de Velde (251). The significance and

probable importance of leucocidin in staphylococcal infections has been repeatedly suggested, and tacitly assumed, but, as pointed out by Holman (126), technical difficulties have hampered its study. A common method of approach has been by means of the familiar "bioscopic" test, introduced by Neisser and Wechsberg (190). Recently Valentine (248) has employed a microscopic method, observing the destruction of leucocytes directly in a series of slide preparations stained with a suitable blood stain.

Valentine's work, which has been confirmed by Proom (207), indicates that the action of leucocidin must be distinguished from that of  $\alpha$ -hemotoxin. According to Valentine, "true" staphylococcal leucocidin destroys both human and rabbit leucocytes, but has little or no effect upon the erythrocytes of either species (248). On the other hand,  $\alpha$ -hemotoxin has essentially no effect on human red or white cells, but destroys both red and white cells of the rabbit. The microscopic picture differs according to the destructive agent. Human and rabbit leucocytes in contact with leucocidin become spherical, the nuclei fragment, and the granules are arranged circumferentially in the cell. The leucocytes may eventually burst, and this invariably occurs when a dried stained film is prepared. Rabbit white cells destroyed by  $\alpha$ -hemotoxin retain the granules grouped in one part of the cell, and remain intact even in the dried film.

It thus appears that these differences in technique have led to differences in interpretation of the exact nature of the leucocidin. It may readily be understood why those investigators using the bioscopic method have considered leucocidin and  $\alpha$ -hemotoxin as identical, with similar properties and endpoints of titration (106, 263). Some differences in properties, and an entire lack of correlation of hemolytic and leucocidic titers have been reported following the use of the microscopic method (197, 207, 248).

The differences in technique must be kept in mind in evaluating reports upon leucocidin. Proom (207) has emphasized that the cultural method of preparing the leucocidin affects the significance of bioscopic titrations. It would appear that the Neisser-Wechsberg technique would be applicable only to the

titration of filtrates containing no appreciable amount of  $\alpha$ -hemo-toxin. For the titration of hemolytic filtrates, Valentine's method, using human cells, is indicated.

It has been generally assumed that the majority of pathogenic staphylococci produce leucocidin. Production of leucocidin has been reported in 17 of 22 strains by Panton and Valentine (197), and in 27 of 36 strains by Valentine (248). All strains in both series were from pathologic sources. All of 26 pathogenic strains were reported by Wright (263) to produce leucocidin (bioscopic method), while 7 strains from non-pathologic sources produced none. Leucocidin titers have been variously reported as ranging from 0.01 to 0.0007 ml. (106), and from 1:200 (263) to 1:500-1:1000 (55, 248).

Leucocidin is antigenic. The production of antileucocidin in rabbits was reported by Denys and Van de Velde (55), Gross (106), and Neisser and Wechsberg (190); its production in horses was described by Panton, Valentine, and Dix (198) and by Wright (263). Titrations of antileucocidin have presented serious technical problems, particularly when attempted by the bioscopic method. No accepted standard for antileucocidin is available, making it necessary for individual laboratories to adopt standards of their own.

A phagocyte-depressing substance produced by staphylococci has been described by Pike (205), in confirmation of earlier work by Hektoen (117) and by Wadsworth and Hoppe (253). It differs from leucocidin in its properties; it is nonspecific, non-antigenic, and cannot be considered as being identical with leucocidin.

#### COAGULASE

The ability of staphylococci to coagulate blood plasma has been recognized and studied at intervals since the demonstration by Loeb (171) in 1903 that broth cultures of *S. aureus* were capable of coagulating goose plasma. The development of a clot in blood broth cultures from patients with staphylococcal septicemia is familiar to all clinical bacteriologists. The relationship of coagulase to pathogenic staphylococci was noted by Much (184),

and its value in differentiating between pathogenic and nonpathogenic staphylococci has been suggested by several investigators (34, 44, 50, 102, 147).

Among the pathogenic cocci, the ability to coagulate plasma is confined to the staphylococci. A variety of other bacterial species has been found not to coagulate plasma (44, 72, 106), although occasional strains of *Pseudomonas aeruginosa*, *Serratia marcescens*, *Escherichia coli*, and *Bacillus subtilis* have been reported to give a positive reaction (72, 171). Strains of both *S. aureus* and *S. albus* will coagulate plasma, although the coagulating power of *S. albus* strains, as a group, is less marked than that of the *S. aureus* strains (27, 34, 72, 102). The plasma-coagulating property of staphylococci appears to be quite stable, and to be retained longer than such properties as chromogenesis and production of hemotoxin (11, 35, 44, 113).

In general, rabbit or human plasma is most readily clotted, one or the other being sometimes reported as being more satisfactory (44, 94, 256), or both being found to be equally good (72). Occasionally plasma from other species, particularly dog plasma, may be most readily clotted by certain strains (100, 104, 204). In the coagulase test, any amount of clotting is considered positive. There is no relation between the rate of clotting and the virulence of the strain.

The presence of coagulating substance in filtrates, originally denied by Kleinschmidt (152), was demonstrated by Gross (103, 104), and has recently been observed by Fisher (72), Cruikshank (44), and Walston (256). Not all filtrates are active, although coagulation may be obtained with broth or agar cultures of strains yielding inactive filtrates.

A distinguishing feature of coagulase is its resistance to heat, exhibited both by cultures and filtrates (72, 108, 256). First noted by von Gonzenbach and Uemura (94), the later report by Gross (105) that it resisted heating to 100°C. has been confirmed by several others. Fisher reported that the thermostability varied with the strains tested, from destruction at 60°C. to only partial destruction at 100°C. for one-half hour (72).

Experiments with the common adsorbing materials were in-



conclusive (72, 87), but it appears that coagulase may be adsorbed on certain types of filters, Berkefeld "V" and "N" allowing passage of the greatest amount of coagulase (72, 87, 249, 256). Fisher called attention to the fact that an amount of calcium sufficient to clot plasma may be washed from both Seitz and Berkefeld filters (72). Thorough washing of filters with normal sterile saline obviates this factor.

The active principle of coagulase may be precipitated from a filtrate by alcohol, acetic acid, or half-saturation with ammonium sulfate, although some loss or destruction may occur (72, 256). The heat-resistance of the alcohol-precipitate is like that of the filtrate (72), while the acetic acid precipitate is less resistant to heat and to storage (256). The active principle may be dialyzed through cellophane after precipitation with alcohol, but not before (256), nor does it dialyze through collodion (87, 249).

*Gratia* (100) demonstrated coagulase in the filtrate of a culture that had been lysed by bacteriophage. This could not be confirmed by Pijoan (204). Fisher obtained results similar to those of *Gratia* with two strains of staphylococci, but with eight other strains there was either considerable loss of coagulating power or complete inhibition (72).

The antigenicity of coagulase is uncertain. The existence of an anticoagulase has been suggested, but it is largely hypothetical, and has not been conclusively demonstrated. Human or horse sera containing demonstrable antihemotoxin do not inhibit coagulase (44, 108, 147). Attempts at immunization with an active filtrate have not been successful (105, 106, 240, 256). The chief evidence suggesting the possibility of inhibition of coagulation by sera has been inferred from occasional samples of blood from cases of human infection which were slow to coagulate (108, 256). However, Sudhues and Schimrigk (240) and Cruikshank (44) were unable to demonstrate any such alteration in coagulability of plasma from cases of staphylococcal infection.

Fibrinogen solutions are clotted by coagulase, but this usually takes place more slowly and less completely than with whole plasma (72, 88, 96, 97, 256). The action of thrombin is suggested, but coagulase is unaffected by "antithrombin" such as hirudin and heparin (72, 98, 256).

## FIBRINOLYSIN

The ability to liquefy fibrin, which has been widely studied in hemolytic streptococci (246), is also possessed by some pathogenic staphylococci. Important differences exist, however, between staphylococcal and streptococcal fibrinolysins. In contrast to the rapid action of the latter, which is often a matter of less than one hour, staphylococcal fibrinolysin often requires several days to bring about dissolution of a plasma clot, and many hours to liquefy a clot obtained from fibrinogen solution (73). A serologic distinction between staphylococcal and streptococcal fibrinolysins has been made (173, 194). When many hours of incubation are required to demonstrate fibrinolysis, complicating factors are introduced by the retraction of the clot from the sides of the tube, and by the tendency of fibrinogen clots to spontaneous lysis (246). Of less importance in the more rapid streptococcal fibrinolysin tests, these factors may seriously interfere with the interpretation of staphylococcal fibrinolysin tests. A slow liquefaction of plasma clot by certain bacterial contaminants (*B. subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Alkaligenes fecalis*, and diphtheroid bacilli) has been described by Fisher (73). Tillett (246) has suggested that this might be the result of proteolysis.

Relatively few strains of staphylococci from human pathologic sources were reported to dissolve plasma clot by Tillett and Garner (247), Fisher (73), and Neter (194). A larger proportion was found by Fisher to dissolve fibrinogen clots, and Aoi (3) reported that 88 per cent of staphylococci dissolved Congo-red-fibrin. The fibrinolytic capacity of pathogenic staphylococci is confined almost exclusively to strains from human sources. None of 24 veterinary strains studied by Madison, and Madison and Dart was active against human fibrin, and all but two of these were inactive against fibrin from a variety of animals (173, 174). These two, isolated from abscesses in horses, dissolved only horse fibrin.

Madison (173) found that 90 per cent of strains from "internal human lesions" (septicemia, osteomyelitis, empyema, cellulitis) were fibrinolytic, while 77 per cent from superficial lesions (acne, boils, nasal sinus, etc.) were inactive against human fibrin.

When tested against animal fibrins, the human strains were found to possess varying lytic capacities; of the active strains, about one-half dissolved the fibrin of only one species, and the remainder were active against two or more fibrins (173). Throughout their work Madison, and Madison and Dart used serum-free fibrinogen clot, with precautions calculated to avoid the complications imposed by this method.

Fibrinolysin has occasionally been obtained in culture filtrates, has been precipitated out with alcohol or acetone (3, 73), and subsequently concentrated (73). Madison, and Madison and Dart concentrated unfiltered broth cultures by precipitation with alcohol, in a manner previously employed by Madison for streptococcal fibrinolysin (172).

Aoi reported the production of antifibrinolysin by the intravenous injection in a rabbit of an "autolysate" or an acetone-precipitate (3). This does not appear to have been confirmed by others. Neter (194) has described inhibition of fibrinolysin by commercial staphylococcal antitoxin, and by the serum of one patient with osteomyelitis.

In common with several other bacterial species, staphylococci produce an anticoagulant, which must be distinguished from fibrinolysin (194). It inhibits coagulation of both human and animal plasma, is produced in broth containing fermentable carbohydrates, but not in plain broth, and is not antigenic. Tillett (246) has suggested that the anticoagulating activity of some bacteria, e.g., *Streptococcus viridans*, pneumococci, may be related to the pH of the medium or to products of hydrolysis of carbohydrates.

#### ENTEROTOXIN

In recent years outbreaks of food poisoning due to staphylococcal contamination of foods have been recognized as being clinically distinct from other types of food poisoning. Dolman (60) cites several such outbreaks involving at least 500 persons in the five years up to 1934. Since then several more outbreaks have been reported, involving about 2000 more individuals (17, 38, 39, 43, 45, 46, 47, 53, 61, 116, 131, 132, 135, 186, 187, 196,

208, 215, 227, 232, 241, 251). The geographic distribution of these outbreaks is of interest, for practically all to date have been in the United States and Canada.

A variety of foods has been incriminated in staphylococcal food poisoning. Chief among these are bakery goods (17, 38, 39, 42, 45, 53, 61, 131, 132, 186, 196, 208, 227, 260), and milk and ice cream (5, 43, 187, 215, 232, 233, 241). In addition, single outbreaks have been traced to such foods as cheese (131), meat and gravy (46, 135, 252), salads (116), oysters (175), and fish cakes (145).

The clinical symptoms of staphylococcal food poisoning include rapid onset (usually two to four hours after consumption of the contaminated food), vomiting, diarrhea, nausea, dizziness, and prostration. Recovery is usually rapid, and the individual is generally quite normal twenty-four hours later. No fatalities have been recorded. A fatal case, reported by Blackman (9), which began with the symptoms of staphylococcal food poisoning, actually involved an acute staphylococcal infection of the small intestine, and septicemia.

Study of enterotoxin has been made difficult because none of the usual laboratory animals is susceptible to oral administration of the toxin. Monkeys and human volunteers have been used, but considerable variation in susceptibility detracts from their value (47, 136, 187, 262). Furthermore, monkeys are rather expensive for many laboratories, and the use of the human subject is often inexpedient. Experiments by Borthwick (16) suggested that the apparent insusceptibility of animals might be due to the pH of the stomach contents. Using the ordinarily refractory guinea pig, Borthwick was able consistently to produce symptoms of food poisoning by adjusting the pH of the stomach contents to 7.3. Dolman, Wilson, and Cockcroft (63) were unable to reproduce these results.

A possible solution to the problem of a suitable experimental animal is suggested in the report of Dolman, Wilson, and Cockcroft (63), who demonstrated typical symptoms of vomiting, diarrhea, and weakness in kittens following the intraperitoneal injection of formalinized or heated toxic filtrates. This has

recently been confirmed by Gwatkin (112), Kupchik (160), Majors, Scherago, and Weaver (175), and by ourselves (unpublished). The intravenous injection of kittens or monkeys has recently been suggested by Davison, Dack, and Cary (51) as possessing certain advantages of sensitivity and economy of materials.

A distinguishing feature of enterotoxin is its thermostability. It is not destroyed by exposure to 100°C. for 30 minutes. Thermostability has recently been questioned by Rigdon (224), who was unable to produce symptoms in puppies or kittens following the intraperitoneal injection of a toxic preparation which had been heated at 100°C. for two hours. Unheated toxin not only caused vomiting and diarrhea, but killed the animals within four hours. It is possible that the extreme length of exposure to heat might readily destroy any toxic factors present, including enterotoxin.

Formaldehyde in the concentration used in the preparation of staphylococcal toxoid has no destructive effect on enterotoxin (63). Jordan, Dack, and Woolpert (134) reported that heating in the presence of N/100 NaOH or N/100 HCl destroys enterotoxin, and that the toxic factor is removed from acid aqueous solution by ethyl ether or chloroform. It is non-volatile and non-dialyzable. It is less readily adsorbed on filters than is exotoxin (262). Storage at low temperatures for 67 days weakens but does not destroy enterotoxin (134).

Human subjects who have repeatedly taken enterotoxin by mouth have shown only a low degree of tolerance (47). Possibly the enterotoxin was destroyed by the digestive juices, or eliminated too rapidly to stimulate antibody-formation, for immunity has been established in experimental animals. Woolpert and Dack (262) produced active, but not passive, immunity in a rhesus monkey, protecting against oral and intravenous administration of enterotoxic filtrates. Dolman, Wilson, and Cockcroft (63) reported the production of active immunity and some degree of passive immunity in kittens. Further evidence of the antigenicity of enterotoxin has recently been supplied by Davison, Dack, and Cary (51).

Reports of Woolpert and Dack (262) and of Dolman (60) appear to show that enterotoxin is a distinct entity, separable from other toxic substances of staphylococci. This is indicated by differences in thermostability and adsorptive capacity, and by serologic evidence. While it is often possible for highly potent exotoxigenic filtrates to be free from enterotoxin (60), it appears that enterotoxin is usually not found in the absence of such exotoxigenic factors as the lethal, dermonecrotic, or hemolytic toxins (60, 63, 160, 262). However, strains of proved enterotoxigenic capacity have been reported which produced no hemotoxin (53, 239, 262).

The symptoms of vomiting and diarrhea are not peculiar to the action of enterotoxin, for, as described above, a characteristic pathologic action of exotoxin in laboratory animals is diarrhea, and both vomiting and diarrhea occurred in the Bundaberg patients. Rigdon (224) noted vomiting in puppies and kittens after feeding sterile nutrient broth. The action of exotoxin may be eliminated by destruction with heat or with formalin (63, 262), or by neutralization with antitoxin (60, 262). Such a filtrate, which originally contained both lethal and enterotoxigenic substances, after suitable treatment produces only gastrointestinal symptoms, with recovery. The pharmacologic action of enterotoxin has not been investigated.

#### *Identification and source of food poisoning staphylococci*

In their biologic and biochemical reactions the staphylococci producing enterotoxin do not show any characteristics distinguishing them from other pathogenic staphylococci (36, 45, 131, 160, 232, 239). Agglutinin and agglutinin-absorption tests provide no basis for differentiation (239). Although the enterotoxigenic capacity of staphylococci may sometimes be restored by growth on starch agar (133), the medium is not differential (232).

In an attempt to identify food-poisoning staphylococci culturally, Stone (56, 235, 236) has proposed media containing gelatin in a beef-extract base, to be used for isolation and differentiation on the basis of gelatin-liquefaction by enterotoxigenic strains. A high degree of specificity was claimed. Toxigenicity of his

cultures was determined by feeding experiments with kittens and by epidemiologic findings. Hucker and Haynes (129) found that enterotoxigenic strains liquefied Stone's medium more readily than the usual nutrient medium. Stone's claims have not been confirmed in several recent reports (37, 63, 112, 160), an important difficulty being lack of agreement between cultural and animal tests. Grubb (111) has emphasized the importance of feeding experiments concomitant with *in vitro* tests to identify enterotoxigenic strains of staphylococci, regardless of source. The conclusions of Chapman *et al.* (36), Hucker and Haynes (129), Chinn (37), and Cogswell, Kilbourne, and Kuhns (39) lose their effect because of lack of concomitant tests. Furthermore, while between 60 and 70 per cent of strains of known food-poisoning origin have been reported to give a positive test on Stone's medium, from 27 to 70 per cent from non-food-poisoning sources likewise react positively (36, 37). The substantial proportion of staphylococci from non-food-poisoning sources which give a positive Stone reaction must be reconciled with the claims for specificity of this *in vitro* method of differentiating food-poisoning staphylococci. The liquefaction of gelatin is of course a property exhibited by many staphylococci.

The source of staphylococci responsible for food poisoning is of considerable epidemiologic interest. The importance of staphylococci from bovine mastitis has been emphasized by Crabtree and Litterer (43), Gwatkin (112), Gwatkin, Hadwen, and Le Gard (113), and Shaughnessy and Grubb (232, 233). Small local supplies of raw milk on farms have frequently been the source of outbreaks, but large urban supplies have not been implicated.

Human sources of contamination are frequently less evident. However, staphylococci of proved enterotoxigenic capacity have been isolated from human throats (239, 262) and infections (160, 239); and in outbreaks of food poisoning pathogenic staphylococci were isolated from persons handling the foodstuffs implicated (145, 187, 227, 260). The conclusion seems to be warranted that outbreaks of food poisoning may be caused directly by contamination of food by staphylococci from human sources.

This possibility has definite epidemiologic implications, and should be carefully investigated.

Dolman (60) has suggested that only a relatively few strains of staphylococci produce enterotoxin, and then only under special conditions of environment (semifluid medium and an atmosphere high in CO<sub>2</sub>) which might rarely be met in foods. On the other hand, experiments by Dack and his associates (49, 146) have indicated that enterotoxic substances may be produced under natural environmental conditions in meat, bread, and cake.

In the control of staphylococcal food poisoning refrigeration of foods is important, but does not solve the whole problem. In controlling staphylococci in bakery goods, the principles of pasteurization have been applied by Stritar, Dack, and Junge-waelter (238) to puffs and éclairs after they have been filled with custard. The bacteria were destroyed, with no alteration in flavor or appearance of the product.

The diagnostic use of agglutinins for staphylococci in the serum of persons with food poisoning was suggested by Shaughnessy and Grubb (233). The presence of such agglutinins in many "normal" persons, however, would seem to invalidate this method, in addition to the fact that the clinical course of the poisoning is usually too short to allow antibody-formation.

#### DIFFERENTIATION BETWEEN PATHOGENIC AND NONPATHOGENIC STAPHYLOCOCCI

The problem of distinguishing between pathogenic and non-pathogenic staphylococci has constantly recurred in investigations on these organisms. Various criteria have been used in the past, including source, chromogenesis, and hemolysis. To these may be added the coagulase reaction and the ability to ferment mannitol, which have received fresh emphasis in recent studies. The term "pathogenic" is, of course, only relative, and has been used somewhat indiscriminately when applied to staphylococci (243).

The source of a staphylococcus may imply pathogenicity only under strictly limited conditions. That presumably nonpathologic sources may yield staphylococci of pathogenic potential-



ity is indicated by the reports of Thompson and Khorazo (243) and of Hallman (114). The former found that 21 per cent of 191 strains from normal mucous membranes fell into a pathogenic group, on the basis of precipitin tests. Hallman found that from 40 to 60 per cent of staphylococci from the normal nose were potential pathogens, as indicated by the coagulase test. Hallman's results have been corroborated by McFarlan (189).

The formation of golden pigment and the ability to hemolyze erythrocytes are frequently associated with pathogenicity of staphylococci. These criteria, particularly chromogenesis, have figured largely in many schemes of classification of these cocci. However, it can no longer be assumed that either criterion, *per se*, is indicative of the pathogenic potentiality of any given strain of staphylococcus. While it is undoubtedly true that the majority of pathogenic staphylococci are of the *S. aureus* type, it is also common experience that there is considerable variation in the degree of pigmentation. Add to this the subjective difficulty often encountered in classifying as *S. albus* or *S. aureus* the borderline strains which produce pale or creamy pigment, and the difficulty of exact correlation of chromogenesis with pathogenicity is apparent. Recent evidence has shown unmistakably that serious attention must be given to *S. albus* strains as possible pathogens (6, 27, 192). That the pigment itself (which resembles carotin) has no relation to pathogenicity or antigenicity was shown by Panton, Valentine, and Dix (198), Goadby (70), and Wright (263).

Dissociation of *S. aureus* into strains of varied pigmentation including *S. albus* strains, was described by Hoffstadt and Youmans (122), and by Pinner and Voldrich (206). The dissociants were nonpathogenic, but Pinner and Voldrich reported the restoration of the *S. albus* type to the virulent *S. aureus* form by prolonged growth in homologous serum. The possibility of restoration to virulence *in vivo* of an avirulent strain is suggested by Hoffstadt and Youmans, and by Pinner and Voldrich. This provokes interesting speculation as to the rôle of such a return to virulence in the lighting up of chronic staphylococcal infections.

Thirty years or more ago a number of investigators reported

some degree of correlation of pathogenicity and hemolysis. More recently von Daranyi (50), Pinner and Voldrich (206), and Chapman and his associates (34) reported similar correlation. This has received fresh emphasis with the repeated reports of the correlation of hemotoxin, dermonecrotxin, and lethal toxin in filtrates of cultures grown under partial CO<sub>2</sub> tension. It must be remembered that such correspondence can apply only to *toxigenic* strains, and that *pathogenic* strains exist which produce no hemotoxin. The lack of correlation of hemolysis on blood agar with the test-tube titration of hemotoxin has been referred to above. In view of this, and in view of the experiences of Parker (201), Flaum and Forssman (76), and Burky (21), described above, it would appear that caution must be used in applying too generally the criterion of hemolysis to pathogenicity of staphylococci.

The value of the coagulase reaction has been established in identifying staphylococci of potential pathogenicity (42, 44, 50, 72, 96, 102, 147). Its close association with pathogenicity is evident from reports of a correlation of better than 96 per cent (33, 44, 114), with a corresponding lack of ability to coagulate plasma on the part of nonpathogenic staphylococci. This correlation, the persistence of the reaction when other *in vitro* properties are lost, and its simple technique make it a readily performed, reliable laboratory procedure of undoubted value.

The fermentation of mannitol as an aid in differentiating pathogenic strains has received repeated confirmation since its use by Gordon (95) in 1903 (32, 33, 44, 65, 112, 114, 139, 243). A high degree of correlation (usually 90 to 95 per cent) with some criterion of pathogenicity has been reported. However, an appreciable proportion of nonpathogenic staphylococci, varying from 11 to 55 per cent (33, 44, 242, 243), has been reported also to ferment mannitol, rendering this test less specific.

Chapman and his coworkers (30-35) have attempted to correlate several *in vitro* tests and to determine their significance as indicators of pathogenicity. Chief among these are pigmentation, hemolysis, and the coagulase reaction, which are interpreted in relation to each other. Good correlation of these tests

with source of the strains has been claimed. On the whole the results of Chapman and his associates serve to bear out the broad relationship between pigment, hemolysis, and pathogenicity which has been assumed by many. That exceptions occur in this general relationship is demonstrated by careful study of their tables and recorded results. The ultimate emphasis on the coagulase reaction is apparent. The limitations of source, pigmentation, and hemolysis as criteria of pathogenicity, referred to above, apply here with equal weight.

Additional *in vitro* methods described by Chapman *et al.* include the "crystal-violet reaction" (30, 31), and the use of brom-thymol-blue agar (35) and phenol-red-mannitol agar (32). Well over 90 per cent correlation of tests in these three media with the major *in vitro* tests of pigmentation, hemolysis, and the coagulase reaction was claimed by Chapman and his associates. The brom-thymol-blue and phenol-red-mannitol media were proposed for isolation. Neither is inhibitory toward other bacteria, and it would appear that the existence of an appreciable number of mannitol-fermenting nonpathogenic staphylococci would detract from the value of the phenol-red-mannitol medium for general use. It may, however, be of particular value in isolating the cocci in cases of chronic conjunctivitis (244).

In papers by Dudgeon (65) and by Winslow, Rothberg, and Parsons (261), the staphylococci are considered to be one large group which includes organisms exhibiting a varied range of biologic and pathogenic capacities. At one extreme is the deeply pigmented, hemolytic, mannitol-fermenting, precipitin-forming, pathogenic *S. aureus*; at the other, approached by the gradual loss of certain characteristics, is the white, possibly non-hemolytic, mannitol-negative, feebly pathogenic *S. albus*, with or without the capacity to form precipitins. A somewhat similar view is proposed by Chapman and his associates (33). According to them, hemolysis, pigmentation, and the coagulase reaction tend to disappear in the order listed, followed later by the other *in vitro* reactions. They have interpreted these variants, or degeneration forms, as possessing varying degrees of pathogenicity, as evidenced by their capacity to react positively to the

*in vitro* tests. It would appear that the evidence supplied by non-hemolytic, unquestionably pathogenic, staphylococci invalidates the application of the term "degenerate" to strains which do not possess certain *in vitro* capacities (53, 239, 262).

In summary, then, a number of *in vitro* reactions have been proposed for the identification of pathogenic staphylococci. While hemolysis and pigment-formation frequently parallel the coagulase reaction, variations occur sufficiently often to render them inadequate, *per se*, and only suggestive at best. Based upon the evidence in the literature and his own experience with all of these *in vitro* tests, it is the considered opinion of the reviewer that the coagulase reaction alone is a sufficient *in vitro* indicator of the pathogenic potentiality of staphylococci. The fermentation of mannitol supplies a valuable confirmatory test. It would seem that the use of numerous *in vitro* tests serves only to multiply confusion, particularly if one is forced by disagreeing reactions to postulate varying degrees of pathogenicity.

#### SEROLOGIC CLASSIFICATION OF STAPHYLOCOCCI

##### *Agglutinins*

Attempts have been made to differentiate staphylococci by serologic reactions, and to show some correlation between pathogenicity and the groups thus established. Although some of the earlier investigators, beginning with Kolle and Otto (157), reported some differentiation between staphylococci from infectious sources and saprophytic forms by means of agglutination tests, there appears to have been no recent satisfactory distinction of staphylococci by this method.

In 1922 Julianelle (138) and Hine (119), studying 25 and 81 strains, respectively, reported the use of agglutinin absorption to establish three major serologic groups, and two sub-groups of staphylococci. Julianelle reported no correlation of serologic grouping with hemolysis or pathogenicity, but he used the relatively insensitive horse erythrocytes, and recorded very few virulence tests. Hine found a general, but not exact, correspondence of the major groupings with pigmentation and fermentation of mannitol, but he recorded no attempt to demon-

strate animal pathogenicity. Unfortunately in neither of these reports is it definitely certain that reciprocal agglutinin-absorption tests were employed. While suggestive, the validity of their groupings must therefore remain in doubt, for, as pointed out by FitzGerald and Fraser (74), only reciprocal tests can provide accurate means of establishing the identity of organisms.

Seedorf (231) used agglutinin-absorption tests to confirm types previously established by means of complement fixation. Reciprocal agglutinin-absorption tests were used by Stritar and Jordan (239) in a study of 94 strains, and by Hopkins and Barrie (128) with an unstated number. The former reported results similar to those of Hine, but no close correlation of biochemical reactions or of hemolysis with grouping was obtained. Hopkins and Barrie established three groups, one containing frankly pyogenic staphylococci, but considered their results as no more than suggestive of a relationship between grouping and pathogenicity. It is probably significant that in essentially all reports on the use of these tests some strains of staphylococci could not be placed in any serologic group.

### *Precipitins*

From several recent reports it appears that the ability of staphylococci to form precipitins may be correlated with pathogenicity. Dudgeon and Simpson (66) noted this correlation, which also extended broadly to the production of pigment and fermentation of mannitol.

Burky (24) has demonstrated a correlation of precipitinogen with pathogenicity which was found to hold for about 75 strains. He established three groups on the basis of the pathologic effect in rabbits of the intravenous injection of 10-day-old broth cultures or their filtrates. One group was definitely toxigenic, killing rabbits within two days, without abscess-formation. The second group produced no toxin, but killed rabbits in from one to 30 days with abscess-formation. The third group was nonpathogenic. Correlation of pigmentation and hemolysis with grouping was not exact. Only members of Group I stimulated the formation of precipitins, but both Groups I and II

gave precipitin reactions with culture filtrates. Members of the nonpathogenic Group III were inactive. We have been able both to confirm Burky's groupings by means of reciprocal agglutinin-absorption tests, and to correlate the grouping with pathogenicity for rabbits, the coagulase reaction, and the fermentation of mannitol (10, 13).

Julianelle and Wieghard (140-142, 259) studied the possible relation of staphylococcal polysaccharides to serologic grouping. In a study of 16 strains two types were established on the basis of precipitation of the purified carbohydrates by antibacterial sera. The pathogenic group was designated as Type A, and the nonpathogenic group as Type B. The assumption of pathogenicity, based on source of the strains, was confirmed with a few representative strains by animal inoculation by Burky's method. Chemical differences in the type carbohydrates was demonstrated by optical rotation, and by differences in the end-products resulting from hydrolysis. Neither carbohydrate alone stimulated the formation of antibodies. A correlation of types and fermentation of mannitol was later reported (139).

Julianelle's groupings have been confirmed and extended by Thompson and Khorazo (243) in a study of 286 strains, and by Cowan (42) with 157 strains. Their groups A and B correspond to the Types A and B of Julianelle and Wieghard. In both reports new groups were established which represent strains showing serologic differences from Groups A and B, and a heterogeneous group of strains which could not be allocated to specific groups by precipitin reactions. A general correlation of grouping with pathogenic potentiality is indicated by the inclusion of from 75 per cent (Cowan) to 78 per cent (Thompson and Khorazo) of strains from human infections in Group A. These strains likewise exhibited the properties generally considered as indicative of pathogenicity, such as pigmentation, hemolysis, the coagulase reaction, and fermentation of mannitol.

Cowan (42) has proposed a classification of staphylococci based on their biologic activities and precipitin reactions. The biologic classification (146 strains) represents a modification of that proposed in 1907 by Andrewes and Gordon (2), with the addition

of hemolysis. Pigmentation is not considered in Cowan's schema. Those strains causing hemolysis are included in the species *S. pyogenes*, which is further subdivided into varieties  $\alpha$ ,  $\beta$ , and  $\alpha\beta$ , according to whether they produce one or both of the hemotoxins described by Glenny and Stevens. *S. pyogenes*  $\alpha$  and  $\alpha\beta$  strains ferment mannitol. The nonhemolytic strains, which usually do not ferment mannitol, are classified as *S. epidermidis*. A general, but not exact, correlation of the biologic and serologic classifications is observed.

The possible influence of dissociation upon the antigenic relationships of staphylococci has been suggested by the work of Hoffstadt and her associates (120-124). *S. aureus* was dissociated into several rough and gonidial forms, differing from the original strain in biochemical and antigenic reactions, and in virulence. They showed that the smooth, rough, and gonidial strains each possessed a distinct antigenic mosaic, as demonstrated by agglutinin reactions. Specific soluble carbohydrates were produced from both the smooth and rough forms, and recently the changes in structure of the bacterial proteins during dissociation were studied.

In summary, it would appear that a broad division of staphylococci into pathogenic and nonpathogenic varieties may be made on the basis of precipitin reactions. That further subdivision might be possible is suggested by the establishment of groups showing varying degrees of serologic relationship. The necessity of including a number of strains, chiefly nonhemolytic, in one "unclassified" group indicates the heterogeneity of these organisms, which may or may not be clarified by further investigation. It may be that the presence of antigens common to the species or of antigenic mosaics which are not readily distinguishable may interfere with attempts at a finer subdivision of the species.

#### MODE OF INFECTION BY STAPHYLOCOCCI

As a result of the recent work on staphylococci, their toxins have received particular emphasis as important factors in the pathogenesis of staphylococcal infections. Although staphylococci have long been recognized as pyogenic organisms, it has recently been suggested that their pathogenic action is due

primarily to their toxins, rather than to their pyogenic capacity (e.g., 59, 110, 137, 214). On this basis staphylococcal toxoid and antitoxin have received wide therapeutic trial.

From experimental and clinical observations it appears quite possible that staphylococcal toxin may contribute largely to the development of lesions, but care must be used in applying the experimental findings to an interpretation of the pathogenesis of human infections; it must be remembered that rather massive doses of toxin have often been used experimentally. Although lethal toxin produces a dramatic effect in experimental animals, its rôle in human infections is probably subordinate. However, a fatal termination in acute staphylococcal infections may sometimes be quite rapid, with little evidence of invasion by the cocci, and is conceivably due to the lethal toxin. This would seem to be true of the deaths in the Bundaberg series. Although intravascular hemolysis is frequent in animals dying after the intravenous injection of exotoxin (143, 192, 193, 262), it must be remembered that human erythrocytes are relatively insusceptible *in vitro* to staphylococcal  $\alpha$ -hemotoxin, and it appears probable that  $\alpha$ -hemotoxin plays only a relatively minor rôle in this manifestation of the pathogenesis of human infections. The  $\beta$ -hemotoxin likewise does not appear to be related to human pathogenicity, nor is it of particular pathogenic importance for animals (19, 91, 182).

That staphylococcal toxin may play an important part in the development of conjunctivitis was suggested in recent work by Thygeson (244) and by Allen (1), who were able to produce conjunctivitis with certain staphylococcal filtrates. The cocci appear to develop in the conjunctival secretion or on dead cells, but do not involve the epithelium of the conjunctivae (244).

The production of lesions of the joints experimentally by the injection of toxin has been reported by Brunschwig and Jung (18) and by Rigdon (220). In considering the relation of staphylococcal toxin to the development of the conditions described, it should be remembered that similar pictures have previously been reported following the intraarticular injection of various specific and nonspecific substances (148, 149).

Possible rôles have been attributed to staphylococcal coagulase



and fibrinolysin in the establishment or dissemination of infections. This has been suggested on the basis of their demonstrated reactions *in vitro*, but lack conclusive experimental proof. Thus, it has been postulated that coagulase might be responsible for the formation of thrombi (44, 88, 106, 108). In the experimental work of Kellaway, Burnet, and Williams (143), and of Fisher (72), intravascular clotting was not obtained, nor did Fisher find any significant amount of thrombosis in 27 autopsies of cases of septicemia due to *S. aureus*. That fibrinolysin might play a part in liberating infected emboli is suggested by Fisher (73) and by Cruikshank (44).

The pathogenic importance of staphylococcal leucocidin has been repeatedly emphasized, with but little experimental evidence, except in the reports of Panton and Valentine, and Valentine (197, 248), who found a reasonably close association of leucocidin-production with certain types of human staphylococcal infection. It is to be hoped that conclusive proof of the rôle of leucocidin may soon be forthcoming.

Based upon a series of studies on staphylococcal immunity, Forssman (78-85) strongly objects to the emphasis placed upon staphylococcal toxins and antitoxins. According to him, immunity to staphylococci depends on a hitherto unknown antibody, which he describes as the "resistance factor." Following a course of intravenous injections in rabbits of a formalinized vaccine, both active and passive protection were reported against the subsequent injection of lethal doses of living staphylococci. Protective sera contained little or no antihemotoxin or anti-dermonecrotxin. In contrast to the development of other antibodies, the resistance factor reached its maximum effectiveness at a considerably later period.

Kitching and Farrell (150) and Downie (64) could not corroborate Forssman's earlier work. This may be understood in the light of his subsequent work, for their protection tests were apparently done during the interval before the sera began to attain their maximum value. It does not, however, account for the failure of Flaum (75) to protect rabbits by a method almost identical with that used by Forssman. Llewellyn Smith

(170) attributed the survival of rabbits in similar experiments to the antitoxin content of their sera, but Forssman would discount this factor because of the survival of some animals whose sera contained an insignificant amount of antitoxin.

While staphylococcal toxin may figure more largely in the development of infection than has been assumed until recently, it would appear that toxin alone is not the sole etiologic factor. This is emphasized by repeated experimental demonstration that active or passive production of antitoxic immunity does not always confer complete protection against living staphylococci, although the survival time of experimental animals is often prolonged (15, 26, 40, 41, 150, 170, 191, 199, 200, 210-213, 222, 223). Likewise a state of "immunity" does not necessarily prevent the development of localized lesions (79, 84, 170). The experimental results are repeatedly confirmed by clinical observations, particularly in osteomyelitis, where they are supported by the reports of Blair and Hallman (12) and Buchman (20) that an increased antitoxin titer following toxoid therapy in chronic osteomyelitis had no effect on the clinical course of the disease.

It is important not to lose sight of the ability of staphylococci to invade tissue and to establish themselves therein. It is, of course, characteristic of most staphylococcal infections that they tend to localize in the tissue or organ affected, where, within a definitely circumscribed area the injurious action of the cocci and their products may be intense. It appears probable that in certain pathogenic staphylococci this capacity far outweighs any ability to produce toxin.

In a series of studies upon inflammation Menkin (176-180) demonstrated the effect of powerfully necrotizing irritants, among which *S. aureus* was particularly potent, in mechanically blocking off an area of injury, and preventing dissemination of the irritant. That blockage had occurred could be readily demonstrated by the inability of the lymphatics to remove trypan blue or bacteria which were injected into an area of acute inflammation. The dye or bacteria injected intravenously could likewise be fixed at a site of inflammation. These findings were

confirmed by Dennis and Berberian (54). The phenomenon suggests the action of staphylocoagulase (126), but Menkin and Walston reported that staphylocoagulase is unrelated to the substance which induces rapid obstruction of the flow of lymph (181). Menkin suggested that the active principle may be similar to staphylococcal leucocidin since filtrates causing the reaction brought about swelling and vacuolization of leucocytes.

Duran-Reynals (67, 68) has described a soluble "spreading factor" produced by staphylococci and other bacteria, whose action in enhancing infection is similar to that previously found by him in various animal tissues, particularly in testicle. The active principle is non-specific in its action, and is produced by many pathogenic staphylococci, in which it is generally correlated with chromogenesis and hemolysis.

#### CONCLUSION

The past several years have witnessed definite advances in knowledge of the staphylococci, particularly with regard to their soluble toxic substances. Although simplified methods of producing toxin have been developed, staphylococcal toxins have yet to reach a degree of purification where their properties and biologic action may be investigated with little interference from contaminating substances. The identity and interrelationships of the several toxic factors remain to be determined accurately. The exact relation of the toxins to the pathogenesis of staphylococcal infections demands further study. A more intelligent approach to the therapy of staphylococcal infections requires more information on these matters. Attempts at serologic classification, and a correlation of this with pathogenicity, appear to indicate some degree of separation of strains of pathogenic potentiality from the nonpathogenic varieties.

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# THE EARLIER PHASES OF THE BACTERIAL CULTURE CYCLE

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## THE BACTERIAL CULTURE CYCLE AND ITS SIGNIFICANCE

Our knowledge of this subject may be considered to have begun with recognition of the fact that bacteria transferred to a culture medium suitable for their growth exhibit a period of delayed multiplication or "lag." Specific study of the problem dates at least as far back as the observations of Müller (1895). He recognized three phases of the early bacterial culture cycle, lag, logarithmic increase and slackened growth and followed Buchner, Longard and Riedlin (1887) in computing generation times during the logarithmic phase by the formula

$$G = \frac{T \log 2}{\log b - \log a};$$

where T represents the time interval, and b and a the final and initial numbers of cells. In the same year, Ward (1895) in a brilliant and exhaustive study of *Bacillus ramosus* also determined generation times and demonstrated the three fundamental phases of slow acceleration, maximum acceleration and reduced acceleration. Furthermore, he indicated clearly the effect upon the culture cycle of temperature, of light rays from the violet end of the spectrum, of food, of oxygen, of dilution and of antiseptic substances.

The phases of the culture cycle were analyzed in a broader sense

by Rahn (1906) and Lane-Claypon (1909), who recognized four phases, lag, logarithmic increase, stationary population and decrease. McKendrick and Pai (1911) attempted to explain assumed changes in growth rate during the culture cycle as manifestations of autocatalytic reactions, the governing factors at any moment being bacterial numbers and amounts of available nutriment. Chesney (1916) made valuable contributions to our knowledge of the culture cycle, as did Buchanan (1918 and 1925) who recognized seven phases, instead of four,—initial stationary phase, lag phase, phase of logarithmic increase, phase of negative acceleration, phase of maximum stationary population, phase of accelerating mortality and phase of logarithmic mortality.

The lag period was specifically studied by Hehewerth (1901) and Whipple (1901), although the phenomenon had been noted by many earlier bacteriologists in connection with multiplication of bacteria in water samples and in relation to the "bactericidal properties of milk," (for further references, see Winslow, 1928). It was carefully analyzed by Rahn (1906), Coplans (1910) Penfold (1914), Ledingham and Penfold (1914) and Chesney (1916).

That the phenomenon of lag had a biological basis was indicated by Müller (1895) who showed that when cultures of differing ages were used for inoculation into a new medium, the generation times in the new medium differed widely. When the source culture of typhoid bacilli was  $2\frac{1}{2}$  to 3 hours old, the generation time was 40 minutes in the new medium; when the same culture was  $6\frac{1}{2}$  hours old the generation time was 80 to 85 minutes; when the source culture was 14 to 16 hours old, the generation time was 160 minutes. Similarly, Barber (1908) and others showed that when transfer was made from a culture in the logarithmic phase, to the same medium and under the same conditions, the new culture multiplied at once at a logarithmic rate.

It was Müller again, in 1903, who first studied the chemical activity of bacteria at various stages of the culture cycle. He did not draw any conclusions as to the ratio of end-products formed to bacterial numbers but comparison of the various tables in his paper makes it clear that the amounts of carbon dioxide and

hydrogen sulfide per cell must have been much greater in the earlier phases of the culture cycle. This phenomenon was clearly recognized at a much later date by Bayne-Jones and Rhees (1929) for heat production, by Cutler and Crump (1929) for liberation of carbon dioxide and by Stark and Stark (1929a) for acid production.

A third differential characteristic of specific phases of the culture cycle is resistance to various harmful environmental influences. Many early observations showed that very old cultures were characterized by low resistance; but this might be due merely to degenerative changes. Much more significant was the discovery by Reichenbach (1911) that young cultures in the lag and early logarithmic phase were more sensitive to heat treatment than those at the peak of their population curve and that resistance increased again in a late stage of the phase of stable maximum population. Even more striking results were reported by Schultz and Ritz (1910). These authors found that a given heat treatment (53°C. for 25 minutes) killed about 95 per cent of colon bacilli from a 20-minute culture. In slightly older cultures, resistance decreased, so that at 4 hours 100 per cent destruction occurred. Then, resistance increased again so strikingly that 7-hour to 13-hour cultures showed no reduction whatever under the same heat treatment. A markedly low resistance to harmful chemical agents was demonstrated by Sherman and Albus (1923) to be characteristic of the lag and early logarithmic phase and these authors, on the basis of their experiments, developed in a highly fruitful manner the concept of "physiological youth" as applied to the bacterial culture cycle.

A fourth characteristic of the phase of "physiological youth" had meanwhile been described by Clark and Ruehl (1919) and by Henrici (1921 to 1928). These investigators demonstrated that certain early phases of the culture cycle are characterized not only by rapid multiplication, high metabolic activity and low resistance to certain harmful environmental conditions but also by highly characteristic types of morphology, the individual cells being in general much larger than in the maturing culture. Wilson (1926) demonstrated the same phenomena by a comparison

of plate counts with measurements of the opacity of bacterial cultures.

Finally, a fifth differential characteristic was described by MacGregor (1910) and by Sherman and Albus (1923), who recorded that young cultures were more resistant than old cultures to acid agglutination. Shibley (1924) demonstrated that in the early phases, the electrophoretic charge on the cells was much less than at a later period.

The fundamental biological significance of the bacterial culture cycle was perhaps first clearly recognized by Henrici. In one of his earlier papers (Henrici, 1925a) he says:

The cells of bacteria undergo a regular metamorphosis during the growth of a culture similar to the metamorphosis exhibited by the cells of a multicellular organism during its development, each species presenting three types of cells, a young form, an adult form and a senescent form; that these variations are dependent on the metabolic rate, as Child has found them to be in multicellular organisms, the change from one type to another occurring at the points of inflection in the growth curve. The young or embryonic type is maintained during the period of accelerating growth, the adult form appears with the phase of negative acceleration, and the senescent cells develop at the beginning of the death phase.

The same theme was developed in his later monograph (Henrici, 1928) as follows:

The acceptance of this theory demands the acceptance of certain corollaries. If it be granted that the cells of bacteria undergo a metamorphosis of the same kind as that exhibited by multicellular organisms, then it must be granted that to this degree a population of free one-celled organisms, even though those cells have no connection other than the common nutrient fluid which bathes them, behaves like an individual. There has already been accumulated a great deal of evidence of other kinds to support the idea that there is no essential difference, that there can be drawn no hard and fast line, between populations of one-celled organisms and multicellular individuals; that a higher plant or animal is but a population of more highly differentiated cells. But there has been, in the past at least, a tendency to look upon cell differentiation in multicellular organisms as being the result of some

organizing agency peculiar to such individuals. If, however, we find in cultures of micro-organisms where no such governing agency can be supposed to exist, a differentiation of cells, even though very primitive, we are forced to conclude that such is not the case; that the high degree of organization of higher organisms is a result and not a cause of the high degree of cell differentiation.

Acceptance of the validity of this analogy between a bacterial culture cycle and a multicellular organism clarifies very greatly the long conflict between pleomorphists and monomorphists in the field of bacteriology. One group has assumed a bacterial "life cycle" governed by some inherent biological tendencies; the other group denies that a cycle of any kind exists. Both perhaps are wrong and both right. One of us (Winslow, 1935) has pointed out that variations in bacterial morphology and physiology certainly do exist but that their succession is governed by environmental and not automatic inherent factors. Furthermore, this is precisely what occurs with higher forms of life where the organism as a whole forms the environment for its individual cells.

May we not assume then, that with all living cells, the "life cycle"—so far as the individual cell is concerned—is a cycle of simple binary fission. Other phenomena involving change in cell morphology and physiology of a cyclical nature are responses to changing environmental conditions and not the result of any inherent time mechanism. If a unicellular organism shows a definite series of morphological and physiological alterations in response to certain changes in environment which are likely to occur with reasonable frequency in its natural life we may call it a "life cycle" if we wish or we may call it something else. In any case, this is the only kind of life cycle (other than binary fission) which can occur in unicellular and relatively simple multicellular forms. In this sense, the bacteria have life cycles. When we find a more complex and more regular life cycle in the higher plants and animals (relatively independent of external environment), it is because the interrelationships of the complex organism produce a cyclical change in the internal environment which is comparable with the change which takes place in a bacterial culture and which affects the individual body cell very much as the cultural environment affects the unicellular organism. (Winslow, 1935.)

The study of the bacterial life cycle is, then, the bacteriological equivalent of the study of embryology, adolescence, maturity and senescence in the higher forms. It is the purpose of the present article to review in orderly fashion some of the things we know about the earlier parts of this cycle—those included under the Buchanan phases of initial stationary, lag and logarithmic growth. No attempt will be made to cover all the literature, which would be impossible in so vast a field; but certain significant and typical data will be cited in regard to each essential point.

#### THE PHASE OF ADJUSTMENT

Müller, in his remarkable pioneer paper of 1895, pointed out that an inoculum from a young typhoid culture showed a much shorter generation time in a new medium than did an inoculum from an old culture. His conclusions did not deal specifically with the lag period but the length of that period obviously influenced his generation times. This is true of many data cited in the present section. Their use as illustrative of factors governing the initial stationary phase seems justified, however, since it is the length of this phase which chiefly determines early generation times. Hehewerth (1901) made similar observations. Barber (1908), working with a microscopic counting method, first showed conclusively that if transfer be made to an identical medium from a culture in the stage of logarithmic increase both stationary phase and lag disappear and multiplication continues at once in the new medium at a logarithmic rate. Lane-Claypon (1909) Penfold (1914) and Chesney (1916) demonstrated the same phenomena. Buchanan (1928) in his general review of bacterial growth curves stresses the fact that transfer from any phase of the culture cycle to an identical medium is followed by continuance of the phase which had been reached by the parent culture, cultures inoculated from either initial stationary phase, lag phase or logarithmic phase, starting in the new culture where they left off in the old one.

The term "lag phase" is commonly applied to the whole period preceding the onset of logarithmic growth; and Ledingham and

Penfold (1914) and Slator (1917) even attempted to formulate a mathematical expression to describe increase in numbers during this period. Such an analysis does not seem very profitable when one considers what radically different processes are at work. Buchanan (1918) was clearly correct in separating a primary phase of initial stationary population from that phase characterized by increase at a rate less than logarithmic. We prefer, however, to call this entire period "The Phase of Adjustment." Instead of the population being stationary during the first minutes or hours after inoculation it may often show a marked decrease. The essence of this phase of the bacterial culture cycle is the adjustment of the inoculated cells to a new medium. Its course and its length depend on the character of the inoculated cells and the nature of the inoculated medium. We agree in part with Hershey (1939) when he attributes the lag phenomenon "to initially unfavorable conditions of growth"; but we cannot agree that this phenomenon is "quite distinct from any peculiarity inherent in the cells." Whether a given condition is unfavorable or not may very clearly depend on peculiarities inherent in the cells, as shown by many observers, from Reichenbach (1911) to Sherman and Albus (1923).

We do, however, concur with Hershey in his contention that when a bacterial culture is inoculated into a *favorable* medium, an initially slow rate of increase in cell numbers cannot be interpreted as indicating "lag," in the conventional sense of low vitality. Most of the early work on this problem deals with cell numbers only; and, since we know that in the early phases of the culture cycle individual cells are of large size, a slow increase in cell numbers may not necessarily mean a slow increase in bacterial mass. Hershey and Bronfenbrenner (1938) and Hershey (1938) have shown that when source cultures of different ages are used to inoculate a highly favorable medium the rate of increase in bacterial mass and the rate of oxygen consumption per unit of mass in the secondary culture is the same for both young and old source cultures. In a more recent paper, Hershey (1939) describes even more conclusive experiments. He cultivated *Escherichia coli* in peptone beef-extract broth and for source



cultures used 3-hour and 24-hour portions of this broth culture. These portions were inoculated into the same medium and the increase in bacterial mass was measured by a photoelectric nephelometer. Simultaneously, determinations were made of bacterial nitrogen and of rate of oxygen consumption. During the first two or three hours of growth in the secondary cultures, the inocula from the young primary cultures showed a much slower rate of cell multiplication than the inocula from old primary cultures but the same rate of increase in total protoplasmic growth, as measured by either nephelometer readings, nitrogen determinations or oxygen consumption. What actually happens is that the cells from a young primary culture develop just as rapidly in total mass in the secondary culture as do the cells from the old culture; but they divide less rapidly.

Unpublished nephelometer studies made in the Department of Public Health of the Yale School of Medicine fully confirm these conclusions of Hershey, and show that the actual rate of increase of bacterial mass is nearly constant from the time growth actually begins up to the attainment of a maximum population. The "lag" in rate of cell-increase is therefore largely a result of delayed cell-division.

The "lag phase," as ordinarily defined by cell counts, may include two quite distinct phenomena, a period of adjustment, characterized by bactericidal or bacteriostatic processes and a period of normally rapid increase in mass with a low rate of cell division. In a very favorable medium, such as Hershey's, the first period disappears; in a very unfavorable medium the second period disappears. It is difficult to distinguish the two processes if cell-mass determinations are not made, and the results of Müller, Hehewerth, Barber and other investigators cited above were no doubt chiefly determined by delayed cell-division. Other data, however, clearly point to temporary bactericidal or bacteriostatic influences.

For example, injury to the inoculum before its introduction into the new medium will prolong lag, as Penfold (1914) found with prolonged chilling. Sturges (1919) noted much-delayed development of colonies on plates seeded from sewage which had

been partially disinfected by copper or sulphurous acid. Allen (1923) reported similar results (measured by generation times) following heat treatment. Recently, Hollaender and Duggar (1938) found that after exposure of *Escherichia coli* and *Serratia marcescens* to ultra-violet radiation which killed four-fifths of the cells the survivors, when inoculated into broth, showed an initial increase, followed by prolonged lag.

The phase of adjustment, as characterized by a stationary or decreasing population, was demonstrated by the early observations on bacterial changes in water samples (reviewed by Winslow 1928) where the alteration of the environment due to placing a sample in a bottle is usually sufficient to cause a temporary decrease in the mixed bacterial flora present. The fact that the bacterial count of a milk sample drawn from the udder shows a similar decrease was first pointed out by Fokker (1890); and there is an enormous literature on the "bactericidal property of milk," a problem recently discussed by Little (1937). Sherman and Curran (1924) showed that a pure culture of *Streptococcus lactis*, transferred in the stage of rapid multiplication to autoclaved milk, showed no lag but, if transferred to unautoclaved freshly drawn aseptic milk, did show a brief lag of half an hour. Sherman and Cameron (1934) found that inoculation from a peptone medium at 45° to the same medium at 10° or *vice versa* and transfer from a peptone medium to the same medium plus 5 per cent NaCl or *vice versa* all showed a considerable initial mortality in the new culture.

The results of Winslow, Walker and Sutermeister (1932) are of special interest in this connection. The strain of *Escherichia coli* studied by these investigators was cultivated in an aerated peptone medium. The addition of 0.1 M NaCl to this medium led to an initial decrease in numbers (instead of the normal lag, characteristic of the plain peptone) followed by a more rapid logarithmic rise and a higher final peak. The addition of 0.5 M NaCl caused a much greater initial decrease, followed by a delayed rise and a final peak lower than that for plain peptone. The addition of 1.25 M NaCl led to a prompt and continuing decline in numbers. Thus, we may find either a steady increase,

a steady decrease or a decrease followed by an increase, depending on the concentration of salt present.

An extremely important point affecting the adjustment of the organism to its new environment is the influence of the amount of inoculum introduced. Wildiers, as far back as 1901, showed that, even in a supposedly ideal medium, a very small inoculum of yeast failed to develop. He concluded that yeast requires for its development an unknown substance ("bios"), soluble in water, dialyzable, difficult to alter or precipitate but destroyed by incineration. Rahn (1906), working with bacteria, discussed this problem and concluded that heat-stable non-filterable substances formed by the bacteria were necessary for maximal multiplication. Penfold (1914) criticized Rahn's work (which, in fact, did not bear clearly on the lag phase on account of the long time-interval employed). Yet Penfold himself found that very small inocula did increase lag. He considered that lag was chiefly due to the lack of intermediate bodies involved in the synthesis of proteins. Chesney (1916) added the interesting observation that an inoculum of washed cells showed a greater lag than one of unwashed cells. Robertson (1923) discussed the problem from a broad biological standpoint and explained it on the ground of an autocatalytic theory of growth.

Walker (1932) demonstrated, in a particular case, that the length of the lag period can be directly controlled by concentration of carbon dioxide, which Valley and Rettger (1927) have shown to be essential to bacterial growth. Walker found that the multiplication of *Escherichia coli* in a synthetic medium could be indefinitely delayed by aeration of the culture with air free from carbon dioxide while growth could be initiated at any moment by intermitting the removal of carbon dioxide. He concludes that "the phenomenon of lag may be due largely, if not entirely, to the time it takes the culture to build up the CO<sub>2</sub> content of the medium or of the cells themselves to a value essential for growth." These conclusions were extended to a wide variety of bacterial species by Gladstone, Fildes and Richardson (1935).

It may be that carbon dioxide represents the "bios" of Wildiers and the "intermediate bodies" of Penfold. Such a hypothesis

might well explain the influence of the size of inoculum upon lag phenomena. We should be cautious, however, in claiming that this is necessarily the only factor at work. It is probable that it is one important factor and it certainly was the controlling one in Walker's study. Other factors may, however, be involved under other circumstances.

The nutrient value of the medium is, of course, another factor in controlling the early phases of growth. Coplans (1910), for instance, showed that lag was increased by addition of dulcitol to a peptone medium and was greater in unheated than in heated milk. Penfold and Norris (1912) showed that early generation times for *Eberthella typhosa* were increased by decreased proportions of peptone in peptone water and decreased by addition of glucose. Winslow, Walker and Sutermeister (1932) found that, when aerated with CO<sub>2</sub>-free air, growth was easily initiated in presence of peptone while lag lasted indefinitely in a Dolloff synthetic medium.

Hydrogen-ion concentration and oxidation-reduction potential are, of course, among the most important factors governing the rate of bacterial reproduction. Cohen and Clark (1919) showed for a number of bacterial types that there is a broad zone of pH within which rates of logarithmic increase in cell numbers are fairly uniform but that on the borders of this zone, very slight changes in pH produce a marked decrease in reproductive rate. The period of "lag" is more pronounced in alkaline than in acid media. That other factors than pH may enter into the picture is indicated by the fact that the border of acid tolerance is different for acetic and for hydrochloric acid.

Fildes (1929) found that the period required for the germination of spores of *Clostridium tetani* was primarily determined by the reducing intensity of the medium. Dubos (1929) reported that growth of pneumococci, streptococci and staphylococci in meat-infusion broth was dependent on a suitable reduction potential.

Finally, the length of the stationary phase is, of course, directly related to temperature. This was shown for water samples by Whipple (1901) by Müller (1903) by Penfold (1914) and by many

others. A recent study by Anderson and Meanwell (1936) of a milk streptococcus (cultured in milk) showed a lag of half an hour at 42°, one hour at 37°, two hours at 30°, and 26°, and three hours at 20°.

Jahn (1934) has recently reviewed the problem of population growth in the Protozoa and finds essentially similar phenomena. He lists food supply, presence of waste products, pH, temperature, CO<sub>2</sub>, oxygen tension, oxidation-reduction potential and light as the chief factors influencing development.

#### THE PHASE OF PHYSIOLOGICAL YOUTH

If the medium be potentially favorable, a time at last arrives when the process of adjustment between inoculum and medium is complete, either by selection of better adapted cells or by accumulation of carbon dioxide or other necessary substances. At this point the initial phase of stationary or decreasing population ceases and the phase of physiological youth begins. The change will not, of course, occur simultaneously with all the cells present in a culture. If we may visualize a given individual cell at this point it will presumably have the general characteristics of the original inoculum. We may assume that the inoculum was taken from a parent culture in the phase of maximal population, since that is the condition under which a complete life cycle is manifest. Under such conditions the culture in its early lag phase will have the following general properties.

The cells will be characterized by relatively low physiological activity (Martin, 1932; Mooney and Winslow, 1935; Huntington and Winslow, 1937); relatively small size (Bayne-Jones and Sandholzer, 1933; Clark and Ruehl, 1919; Henrici, 1928; Jensen, 1928); low multiplication rate (Rahn, 1906; Coplans, 1910; Lane-Clayton, 1909; Penfold, 1914; Ledingham and Penfold, 1914); rather high resistance to unfavorable conditions (Schultz and Ritz, 1910; Sherman and Albus, 1924; Elliker and Frazier, 1938); and relatively high electrophoretic mobility (Moyer, 1936).

As soon as the process of adjustment is completed, however, the cell in its new medium passes into a phase of physiological youth,

characterized by active metabolism and rapid increase in mass but—at first—with delayed cell-division.

The morphological and physiological manifestations of youthful activity are nearly simultaneous. Huntington and Winslow (1937), however, have shown that with *Escherichia coli*, *Salmonella pullorum* and *Salmonella gallinarum* in an aerated medium, both the initial increase and the peak of metabolic activity slightly precede the initial increase and peak in cell size. In eight sets of experiments with these three organisms in three different media, the ratio of cell size for the one-hour culture as compared with the 24-hour culture was close to unity in six instances and about 2 in the other two instances. The corresponding ratio for metabolic activity was unity in two instances and from 3 to 16 in the other six instances. Martin (1932) considers increases in cell size and metabolism to be simultaneous, but since his "simultaneous" increases represent observations at the end of a given time interval in the case of cell size and cumulative results of the entire corresponding interval in the case of metabolic activity, his results, too, fit the theory that increased metabolic activity is the first evidence of physiological youth.

### *Metabolic activity*

The generally high metabolic activity for the early phase of the culture cycle was first indicated in the remarkable study of Müller (1903). He does not give strictly comparable figures for cell numbers and metabolic activity, but does show that carbon-dioxide production, H<sub>2</sub>S-production and formation of products of nitrogenous decomposition all reach a high peak in the early phases of the culture cycle. If one may assume that, in these metabolic studies, the rates of multiplication were the same as those given by Müller in his other tables it is clear that metabolic activity per cell was enormous during the early lag period.

Bayne-Jones and Rhees (1929) were perhaps the first investigators to provide actual data on metabolic activity per cell per hour at different periods of the culture cycle. They studied heat production in cultures of *Escherichia coli* and *Staphylococcus*

*aureus*. Their most striking results were for *E. coli* in peptone broth, where the gram-calories of heat produced per cell were  $60 \times 10^{-11}$  at one hour, 198 at two hours, 130 at 3 hours and 75 at seven hours; but their other experiments indicated the same general relationship. With *E. coli* in plain broth, the increase was more than eightfold. Wetzel (1932) presented a formal mathematical analysis of the data of Bayne-Jones and Rhees and concluded that the formulae involved described equally well the course of heat production in a bacterial culture and in the growing human body. Schmidt and Bayne-Jones presented similar data for *E. coli* in peptone water, which yielded values of  $232 \times 10^{-11}$  gram-calories per cell for the first hour, 194 for the second hour, 51 for the third hour and 4 for the sixth hour.

Similar data for oxygen consumption next became available. Burk and Lineweaver (1930) showed for *Azotobacter* that rate of oxygen consumption per unit rate of increase in cell numbers was greater during the first nine hours of the culture than for the ninth to the twelfth hours. Eaton (1931), working with staphylococcus cultures, reported a higher respiration rate during the first two and a half hours. Gerard and Falk (1931) provided the first definitely quantitative data for *Sarcina lutea*. They computed a consumption of 6.5 cu.mm. of oxygen per milligram dry weight of culture for the early stages of the culture cycle as compared with 2.6 in the phase of stable maximal population. They did not, however, interpret the phenomenon as a manifestation of physiological youth but attributed it to a partial asphyxia produced in the preparation of the inoculum. Martin (1932) saw the problem in its true setting, although he did not compute rates per cell. He noted, however, that the rate of oxygen consumption reached a peak for *Escherichia coli* between 30 and 90 minutes while cell size was greatest at 60 to 120 minutes.

The fact that Hershey (1939) did not detect any change in rate of oxygen consumption at different periods of the culture cycle is not surprising since the range of ages covered in his secondary cultures was only from 1.2 to 2.3 hours. What he does show, however, is that this rate—under the conditions of his experiment—was not affected by wide variations in the age of the

*primary* culture from which this secondary culture was inoculated. This latter conclusion, we are quite ready to accept. As pointed out above, the physiological state of a primary culture influences the rate of growth in mass in a secondary culture chiefly through its influence on the phase of adjustment—and, in Hershey's case, the medium was so favorable that the phase of adjustment practically disappeared.

More complete information is available with regard to CO<sub>2</sub> production. Cutler and Crump (1929) reported that liberation of CO<sub>2</sub> in sands and soils was greatest per million bacteria when the numbers of bacteria were low. Walker and Winslow (1932), working with *Escherichia coli* in an aerated culture, reported 41 to 185 mg.  $\times 10^{-11}$  CO<sub>2</sub> per cell per hour formed in the lag period against less than 2 mgm. for the close of the logarithmic phase. Walker, Winslow and Mooney (1934) studied the problem in media aerated with nitrogen instead of air and found that, under such anaerobic conditions, CO<sub>2</sub>-production per cell per hour in a peptone medium fell from 42 mg.  $\times 10^{-11}$  in the first hour to 27 in the second hour, rose to 68 in the third hour and then fell. Under anaerobic conditions in peptone-glucose the figure fell from 42 in the first to 36 in the second hour, rose to 211 in the fourth hour and then fell. The second-hour temporary decrease seems to be a peculiarity of the anaerobic state and the enormous rate recorded for peptone-glucose indicates anaerobic utilization of sugar. No increase of CO<sub>2</sub> production per cell was ever noted in the earlier work under aerobic conditions in sugar-containing media, as a result of the presence of the sugar, in spite of the fact that sugar was actively fermented. Walker, Winslow, Huntington and Mooney (1934) worked with *Escherichia coli* in various media and reported maximal production of CO<sub>2</sub> (117 to 123 mg.  $\times 10^{-11}$  per cell per hour) during the second hour of the culture cycle (late lag and early logarithmic phase) falling to 16 to 22 after five hours (beyond the close of logarithmic phase). Mooney and Winslow (1935) studied *Salmonella gallinarum* and *Salmonella pullorum* as well as *E. coli*. A high peak of metabolic activity during the lag and earlier logarithmic period was always apparent. In this early phase,



79 to  $145 \times 10^{-11}$  mg. of  $\text{CO}_2$  was formed per cell per hour as compared with 2 to 8 in the stage of maximal population. *Salmonella pullorum* in peptone-glucose had a very long lag period and the figures in table 1 give a valuable slow-motion picture of the processes involved.

Where the process is more rapid, the various stages are telescoped, so that a first-hour observation often shows a high metabolic rate; but such an experiment as that cited gives us a true picture of the processes at work.

In all the studies cited (except that of Gerard and Falk) the high metabolic rates of the early growth phases were computed

TABLE 1

AGE OF CULTURE	BACTERIA	CO <sub>2</sub> -PRODUCTION PER CELL PER HOUR
hours	millions per ml.	mg. $\times 10^{-11}$
1	12	6
2	10	30
3	13	33
4	16	99
5	39	114
6	70	96
7	320	57
8	603	26
9	706	17
25	332	8

per cell; and these high rates of activity might be in part due to the larger size of the cells. It was clear from consideration of the magnitude of the changes involved that this could not explain the whole phenomenon; but the relationships of cell size and metabolic activity were finally clarified by Huntington and Winslow (1937). These observers combined simultaneous data on cell numbers, cell size and metabolic activity, working with *Escherichia coli*, *Salmonella gallinarum* and *Salmonella pullorum* in aerated cultures and using three different media. They computed rates of  $\text{CO}_2$ -production per cubic micron of bacterial substance and found that maximum values for the lag and early logarithmic phases ranged from  $86$  to  $216 \times 10^{-11}$  mg. per cubic

micron while for the phase of stationary maximum population the corresponding figures varied from 5 to 19.

A fourth measure of metabolic activity, for which similar evidence is available, is acid production. Stark and Stark (1929a) found the rate of fermentation of *Escherichia coli* to be  $4.6 \times 10^{-11}$  mg. per cell per hour for young cells and 0.9 for old cells. Less striking but similar relationships for fermentation by *Streptococcus lactis* have been reported by Rahn, Hegarty and Deuel (1938).

Liberation of  $\text{NH}_3$ -nitrogen is a fifth type of metabolic activity associated with physiological youth. Here, there arises the question whether lowered values for  $\text{NH}_3$ -nitrogen in later growth phases may not be due to more rapid utilization, rather than to a lessened rate of liberation of  $\text{NH}_3$ . This question cannot be categorically answered, but the close parallelism between  $\text{NH}_3$  and  $\text{CO}_2$  suggests that both are examples of the same phenomenon. The first suggestion of such an effect in the case of  $\text{NH}_3$  (after the pioneer work of Müller) came from Meiklejohn (1930) who studied the relation of numbers and  $\text{NH}_3$ -production in a peptone culture of a soil bacterium. Number of cells and efficiency in  $\text{NH}_3$ -production showed an inverse relationship. Walker and Winslow (1932), in a more detailed study, determined the rate of  $\text{NH}_3$ -production for *Escherichia coli* in the lag phase in different media as 6 to 36 mg.  $\times 10^{-11}$  per cell per hour while the corresponding figure for the phase of maximum population was 0.2 or less. Walker, Winslow, Huntington and Mooney (1934) reported that at  $1\frac{1}{2}$  hours (late lag phase) the  $\text{NH}_3$ -nitrogen yield varied in different media from 26 to 50 mg.  $\times 10^{-11}$  per cell per hour while after 5 hours (post-logarithmic phase) the values fell to 3 or less.

Hewitt (1937) reports electrode-potential curves for various organisms at various phases of the culture cycle. With hemolytic streptococci in broth the  $E_h$  begins to fall after 30 minutes and drops to a minimum in 12 hours (approximate end of the logarithmic phase). Subsequently the value rises or (with the diphtheria organism) may remain at a low level. In aerobic

glucose-broth, the streptococcus shows a sharp rise after five hours (Hewitt 1929). The phenomena involved are, however, too complex to be related with certainty to the physiological culture cycle.

Child (1929), in his striking essay on Senescence and Rejuvenescence, points out that phenomena exactly like those described above are manifest in the life history of some of the simpler invertebrates (hydroids and planaria). From the very early stages of the life of these multicellular organisms there is manifest a progressive decrease in oxygen consumption, carbon dioxide production and growth rate. Again, the analogy between a bacterial culture cycle and the life of a multicellular organism is strikingly illustrated.

### *Morphological changes*

In parallel with the outburst of metabolic activity which characterizes the early lag period,<sup>1</sup> and almost—but not quite—simultaneous with it, come fundamental changes in size and other morphological characteristics of the bacterial cell.

Many of the earlier workers in bacteriology noted the presence of large cells in the initial phases of the culture cycle (see review by Ward, 1928). One of the most significant of such observations was that of Fuhrmann (1908; 1926) who observed that an organism which he called *Pseudomonas cerevisiae* exhibited a rather regular series of morphological phases in various media, beginning with small rods and passing on to large swollen cells and thread-like forms with refractive points and stainable granules. The first exhaustive study of this problem, with clear emphasis on the time-relations involved, was, however, that of Clark and Ruehl (1919).

These investigators studied 70 strains belonging to 37 species of bacteria and found that in all cases, except certain corynebacteria and the glanders organism, marked increase in size occurred

<sup>1</sup> These changes are commonly attributed to the *late* lag phase; but this is because under the term "lag phase" there is also included the "initial stationary phase." Using Buchanan's more penetrating analysis the characteristics discussed are associated with the very early lag phase.

in the early stages of the culture cycle. This increase generally manifested itself after 2 hours and the maximum size was as a rule noted between 4 and 6 hours. In a later review, Clark (1928) says "During the logarithmic period when maximum reproduction occurs, the cells from the young cultures of many genera of bacteria attain their maximum size, two to six times larger than the cells from the twenty-four-hour parent-cultures." During later progression, up to 18 to 24 hours, toward a stationary period the bacteria became gradually smaller in size. The large cells stain more intensely than those of normal size.

The major contribution to our knowledge of this subject came from Henrici who published a remarkable series of papers, and finally a book on the subject of Morphologic Variation and the Rate of Growth of Bacteria,<sup>2</sup> between 1921 and 1928. In his first paper (1921) he showed for a spore-bearing aerobe that the cells began to increase in size in the lag rather than the logarithmic phase, reached their maximum dimensions shortly after the beginning of maximum multiplication (six times those of the original inoculum), then gradually becoming shorter. *Escherichia coli* (1924a) showed maximum size at three hours (about the middle of the logarithmic phase)<sup>3</sup> and was down again to normal by 6 hours (end of logarithmic phase). When the cells are largest, intracellular granules disappear, the protoplasm becomes more hyaline and stains more deeply. If transferred at the moment of increasing cell size, increase proceeds; if at the moment when original size has been reached, increase begins at once; if transferred later, lag occurs (in cell size increase). The richer the medium, the longer is the period of size increase and the greater the maximum size attained (1925a). The cholera vibrio develops oval cells of less than normal curvature in the lag phase. As the logarithmic phase sets in, the cells become elongated and curved again (1925b). In Henrici's final monograph (1928) he reviews all this material with full data as to the curve of distribution of cell length and form at each stage. The phase of large

<sup>2</sup> Passages from this work are cited below through courtesy of Charles C. Thomas, Publisher, Springfield, Illinois.

<sup>3</sup> Henrici would probably have found even larger cells at a still earlier period.

cells is clearly associated with greater variability in size. The more rapid the growth, the greater is the cell size. The presence of sodium ricinoleate tends to produce very elongated cells while  $\text{CaCl}_2$  (which raises surface tension) has an opposite effect. Large cells of *Escherichia coli* are more stainable and perhaps show an isoelectric point further to the acid side.

In general comment, Henrici says:

These three correlated properties: Increased length and slenderness of the cells, indicating a greater magnitude of some axially disposed force opposing the surface tension of the medium; increased intensity of staining with basic dyes and decreased susceptibility to acid agglutination, indicating an isoelectric point of the protoplasm more on the acid side; and increased susceptibility to injurious agents, all serve to distinguish the young, actively growing cells from the resting cells, and justify our recognizing these long cells as a distinct morphologic type, as *embryonic* cells, characteristic of the growth phase of the culture.

The general significance of the phenomena involved are discussed as follows:

It would seem from my data that the division of the growth curve into a lag phase (of accelerating growth), a logarithmic growth phase, and a resting phase, is not so significant as a division into a phase of accelerating growth and a phase of negative acceleration in growth; the so-called logarithmic growth phase when present is but a long drawn out point of inflection. For the morphologic variations which occur during the early stages of growth progress definitely to this "mid-point" (as Pearl designates it) of the growth cycle, then turn sharply in the opposite direction. The embryonic forms reach their maximum development just at the beginning of negative acceleration in growth rate, the mature forms at the end of growth.

These embryonic forms will vary in their characters with different species of bacteria, but it is apparent from what has been presented here, as well as from the observations of Clark and Ruehl, that with most forms, especially the rods, they differ from the mature forms particularly in increased length and slenderness. The diphtheroid group are apparently an exception, the embryonic forms being shorter and more nearly approaching the spherical form. In all cases these embryonic forms seem to possess a higher affinity for the basic aniline

dyes. In those cases where the young cells show an increased size there is also apparent an increased variability in size. In those forms which develop intracellular granules or other structures these are lacking in the embryonic cells. In the case of the cholera vibrio, which may perhaps be taken as a type of the spiral organism, the embryonic forms are characterized particularly by straightness of their cells; they are bacillary in form.

The mature or differentiated forms, beginning to develop with negative acceleration in growth and reaching their maximum at the end of growth, are just the reverse of the embryonic forms in the characters enumerated above; . . . It is of course obvious that these cells are not differentiated in the sense that different cells show a great diversity of form and internal structure as occurs in the differentiated cells of a multicellular organism; such cannot be the case because the cells are all contained in the same environment which must be nearly uniform throughout. But the individual cells do show a differentiation in their internal structure, many forms developing within the protoplasm spores or granules of one type or another, especially volutin. Now it is just this development of internal "paraplasmatic" structures which characterizes the differentiated cells of a multicellular organism, and which are either the result or the cause of their diversified function. In this sense at least, then, there does occur differentiation in the mature cells of bacteria. (Henrici, 1928.)

It should be noted that the diphtheria bacillus seems to offer an exception to the general rules which operate with the other organisms studied. Albert (1921) recorded an early increase in size even for this organism but Clark and Ruehl (1919) found the young cells here smaller than in a later resting period. Henrici (1922), working with a chromogenic diphtheroid, confirmed Clark and Ruehl's observations but his figures (Henrici, 1928) suggest that the special tendency of this organism to form chains of streptobacilli may be the complicating factor in this case.

Meanwhile, Wilson (1926) has demonstrated increase in cell size during the earlier phases of the culture cycle by an interesting new method. He compared increasing opacity of a culture with cell-counts and found that the number of viable cells necessary to produce a given opacity was five times as great in a 26-hour culture as in a 4-hour culture of *Salmonella aertrycke*. Alper

and Sterne (1933) present similar data. The possible presence of more non-viable cells in the later phases somewhat vitiates these conclusions.

Jensen (1928) made a particularly significant study of the morphology and growth of *Escherichia coli*, with special emphasis on the history of the individual cell. His careful observations led him to the conclusion that the phase of absolute latency is one in which cell size increases without fission. He finds that, when an individual cell has reached its maximal size, multiplication at full logarithmic rate begins, so that the phase of relative latency (increase in numbers at sub-logarithmic rate) is merely a statistical characteristic of the culture as a whole. He gives us a new and very interesting type of information in regard to the proportion of individual cells in a culture which proceed to subdivide within a reasonable period of observation. At two hours (late lag or early logarithmic phase) 100 per cent of the cells exhibit prompt fission, while after four or five hours (end of logarithmic phase) the ratio falls to 10 to 54 per cent. After twelve hours, it rises once more.

The work of Clark and Ruehl and Henrici tended to show that the phase of maximum cell size and that of most rapid multiplication coincide. Jensen's studies, however, strongly suggest that increase in cell size actually precedes a rise in division rate. Thus, his colon bacilli in the first half hour were large but not yet dividing. From one to one and one-half hours (early logarithmic phase) they were large and dividing. At  $3\frac{1}{2}$  hours (middle logarithmic phase) weakly-staining shadow forms began to appear. At five hours (post-logarithmic phase) the shadows had disappeared and cells were small again. These shadow forms were capable of reproduction if transferred to a new medium but apparently could not do so in the original medium.

The next important contributions to this problem came from Bayne-Jones and his associates at Rochester, using a very accurate technique based on measurement of cinematograph records. Working with *Bacillus megatherium*, Adolph and Bayne-Jones (1932), like Jensen, found that fission rates lagged behind rates of increase in total cell protoplasm. They noted

(as had Schmalhausen and Bordzilowskaja, 1926) that the rate of growth of a single cell from fission to fission is approximately constant at a given time but that the mean rate of growth in size per cell in a culture rises sharply to the end of the second hour and falls after the third hour (when an 18-hour inoculum is used). At the peak of increase in cell size the bulk of cell substance doubles every 22 minutes, and the authors compute that if such a growth rate continued for 24 hours a single filament could be produced reaching 4000 times the distance between the earth and the sun. With *Escherichia coli*, Bayne-Jones and Adolph (1932b) found that at the end of the first hour there was

TABLE 2  
*Time of maximum activity (hours after inoculation)*

	SIZE OF CELL	RATE OF CELL MULTIPLICATION
<i>E. coli</i>		
Peptone.....	2	4
Peptone-glucose.....	2	3
<i>S. gallinarum</i>		
Peptone.....	3	5
Peptone-glucose.....	3	6
Peptone-lactose.....	3	7
<i>S. pullorum</i>		
Peptone.....	4	9
Peptone-glucose.....	4	7
Peptone-lactose.....	3	7

a maximum rate of growth in cell size, with no increase in numbers, while the maximum rate of reproduction occurred at the end of the second hour. Bayne-Jones and Sandholzer (1933), with *E. coli*, report that the volume of a cell of the initial inoculum was less than 1 cubic micron. At the end of the first hour this value had increased to 4 cubic micra. At the end of the logarithmic phase (160 minutes) the mean cell size was again about 1 cubic micron.

Huntington and Winslow (1937) have more recently confirmed, once more, the conclusion that increase in cell size precedes the logarithmic phase of multiplication. They studied *Escherichia coli*, *Salmonella gallinarum* and *Salmonella pullorum* in aerated



cultures using various media and found that, with eight combinations of organisms and media, the peak of reproductive activity came from one to five hours later than the peak of cell size, as indicated in table 2.

In five out of these eight instances maximum cell volume was noted during the lag phase, in the other three instances in the early logarithmic phase.

A suggestive check on these time studies is to be found in a paper by Fischer (1932) who observed the development of spreading cultures of *Escherichia coli* in soft agar. He noted in the outer zone of a spreading colony large cells ( $7 \times 2 \mu$ ); in an inner ring, small cells ( $4 \times 1 \mu$ ); and in the center, a region of autolysis. Here are the various time changes of Jensen, manifest at one time in those areas of a culture which are of different age.

Longworth (1938) has recently reported interesting studies of the variations in cell morphology in bacterial cultures as well as in ratios between sizes, counts and total mass as measured by a photoelectric densitometer. Hershey (1939), in the paper discussed in an earlier paragraph, expresses cell size as a ratio between nephelometric count and viable count and finds that when a 24-hour primary culture is used for inoculation the initial ratio is about 1 and increases to 3 during the first hour of the secondary culture and to 6 or 8 during the second hour. Inoculation from a 3-hour primary culture gives an initial ratio of 5 to 6, which is maintained for several hours, showing that in this respect (though not in mass-reproductive rate) the cells of a young primary culture behave differently for a time in a secondary culture from those derived from an old primary culture.

#### *Lowered resistance to unfavorable agents*

A third very important characteristic of the large actively metabolic cells of the early culture cycle is their markedly reduced resistance to various harmful chemical and physical conditions.

This phenomenon was, perhaps, first pointed out by Schultz and Ritz (1910). These investigators, using colon bacilli, exposed cultures of various ages to heat treatment at  $53^\circ$  for 25 minutes. In a 20-minute culture about 5 per cent survived such

treatment. In a 50-minute culture (still in the lag phase) 1 per cent survived. In 4-hour cultures (early logarithmic phase) 100 per cent were killed, and in cultures from 7 to 13 hours old (late logarithmic phase) the same treatment produced no reduction at all. These results would seem almost unbelievable if they had not been so often confirmed. Reichenbach (1911), in the very next year, reported a reduction (caused by exposure to a temperature of 47 to 51° for five minutes) of 71 to 100 per cent in 5 to 8 hour cultures and of only 3 per cent in a 28-hour culture.

The biological importance of this phenomenon was first emphasized by Sherman and Albus (1923) in their striking paper on *Physiological Youth in Bacteria*. These investigators demonstrated low resistance for *Escherichia coli* in the early culture-phases with respect to four independent conditions. They confirmed the general conclusions of Schultz and Ritz and of Reichenbach with respect to heat resistance, (although the differences were not quite so striking). They found that chilling the cultures showed a similar differential, old cultures exhibiting no reduction while young cultures did. They demonstrated the same phenomenon on transfer to a 2 per cent NaCl solution in distilled water and, finally, on exposure to 0.5 per cent phenol. In general four-hour cultures (logarithmic phase) were the ones which showed the low resistance. The results, so far as exposure to heat and NaCl was concerned, were confirmed for a *Proteus* strain. In a second communication Sherman and Albus (1924) worked out the time relations in greater detail. *E. coli* was grown at 37° in peptone-water and at intervals transferred to 5 per cent NaCl. Cells removed after one hour showed no mortality. After 90 minutes (while the parent culture was still in the lag phase) the cells showed definite susceptibility; and after 2 and 2½ hours (when rapid increase in numbers was going on in the parent culture) mortality was greatly increased. The authors point out that this phenomenon gives clear evidence of "biologic rejuvenescence" before active reproduction begins.

The greater stainability reported by Henrici (1928) and others for the phase of increased cell size may be related to the lessened

resistance to chemical agents at this period. A later contribution in this field of resistance to chemical agents was that of Watkins and Winslow (1932) on disinfection by N/100 NaOH at 30°C. The following values for K were reported for cultures of varying age:

Age hours	K
8	.38
11	.22
14	.15
17	.14

Literature as to the Schultz and Ritz phenomena of lowered resistance to heat is voluminous. It was described by Ørskov (1925) for colon-typhoid strains and by Robertson (1927) for *Microbacterium lacticum*, *Sarcina lutea* and *Streptococcus thermophilus*. *S. lutea*, exposed to 71°, showed a 99 per cent reduction in a four-hour culture and no reduction in a post-logarithmic culture. *S. thermophilus*, heated at 63°, showed over 99 per cent reduction in a six-hour culture and only 55 per cent reduction in a 30-hour culture. Sherman, Stark and Stark (1929), and Stark and Stark (1929a; 1929b) made similar observations for streptococci and ropy-milk organisms. Fabian and Coulter (1930), Hammer and Hussong (1931), Heiberg (1932), Dorner and Thöni (1936) added more evidence along the same line. Hershey (1939) inoculated from 3-hour and 24-hour cultures into distilled water and found that while the young cells were completely eliminated after 15 minutes, half of the cells from the old culture were alive after one hour. It should be noted that, in many of these studies, the low resistance phase was contrasted with rather late periods of the culture cycle; so that it is not quite clear whether it is a contrast between youth and maturity or between maturity and old age of a culture with which we are dealing.

There are a few studies which appear to conflict with this considerable mass of evidence. The first of them, by Anderson and Meanwell (1936) does, indeed, seem hard to explain. These investigators worked with a thermoduric milk streptococcus cultivated in milk at various temperatures. At intervals tubes were removed and exposed to a temperature of 63° for 30 minutes.

When the parent culture was grown at 42° the lag period was very short and resistance to heat treatment decreased steadily. At 37° the lag period lasted about one hour and heat resistance rose at half an hour and then fell. In the cultures grown at 30°, 26° and 22°, respectively, lag was of course more and more prolonged but, in each case heat resistance was greatest in the early logarithmic phase. In other words, these results directly contradict all the earlier work of a score or more of different investigators. Somewhat similar results have recently been reported by Claydon (1937).

A third study which apparently conflicts with general experience appears, on closer analysis, not to do so but merely to bring out a very interesting new phenomenon with regard to the

TABLE 3  
*Parent culture at 28°*

AGE	NUMBER OF BACTERIA PER ML.	PER CENT SURVIVAL WHEN HEATED AT 53°
<i>hours</i>		
0	25,000	6.4
1.5	33,800	34.0
3	40,350	1.4
6	297,000	.1
9	730,000	.02
36	3,200,000	5.8

culture cycle. Elliker and Frazier (1938 a and b) worked with *Escherichia coli* grown at 28° and 38° and then heat-shocked at 53° for 30 minutes. The results obtained are best illustrated by table 3.

The usual fall in resistance is shown for the late lag and early logarithmic phase; but the new phenomenon revealed is a temporary brief increase in resistance in the very early lag phase. More careful studies, at shorter intervals during the lag phase, showed that the initial increase in resistance lasted up to the first hour at 28° and from the twentieth to the fortieth minute at 38°. This suggests a new and exceedingly interesting characteristic of the early lag period.

The lowered resistance of the late lag and early logarithmic

phase has been demonstrated with respect to other physical influences than heat and cold by other investigators. Gates (1929) reports that a four-hour culture of *Staphylococcus aureus* is much more readily killed by a given intensity of ultra-violet light than is a 28-hour culture; and Kimball (1938) finds that budding of yeast is inhibited in a magnetic field only in the last half of the lag phase.

Bayne-Jones and Sandholzer (1933) reported that young cells are more readily attacked by bacteriophage than older ones.

A most interesting observation has recently been reported by Hegarty (1939), which may or may not be related to those characteristics of the youthful bacterial cell which make it susceptible to harmful agents in the environment. This investigator found that cells from a mature culture of *Streptococcus lactis* grown in glucose-tryptone broth can not attack galactose, lactose, sucrose or maltose until they have multiplied for some time in the presence of the respective carbohydrates. Cells from a 1- or 2-hour culture, however, attack these new sugars much more promptly. This is in the late lag and very early lag phase and the power of adaptation to the new carbohydrate, thereafter, decreases progressively. The power to utilize new carbohydrates is a favorable one while susceptibility to harmful agents is an unfavorable one; but it is conceivable that both may be related to some underlying property of ready responsiveness to the environment.

#### *Acid agglutination and electrophoretic mobility*

Finally, there is a fifth characteristic of the large and metabolically active cells of the youthful phase of the bacterial culture cycle, which may again be related to their low resistance to harmful chemical agents but is demonstrated by direct physical measurements. This is the property of low susceptibility to agglutination, coupled with low electrophoretic charge.

The first observation of this kind with which we are familiar was made by MacGregor (1910) who reported that meningococci from a two-day culture needed a concentration of 1:10 to 1:40 of normal serum to produce flocculation, while a four-day culture

sometimes flocculated with a concentration of 1:100 and at times even showed spontaneous agglutination. Gillespie (1914), working with pneumococci, found that cultures up to eighteen hours did not agglutinate in 20 hours without salt while, after twenty-four hours, they agglutinated in 6 hours. The reaction favorable to agglutination was less acid for the young cultures. Sherman and Albus (1923), working with *Escherichia coli*, reported that four-hour cultures were not agglutinated by an acidity of pH 3.0 while a twenty-four-hour culture agglutinated at a pH of 3.8.

Shibley (1924) made the first direct observation of electrophoretic charge (with pneumococci and paratyphoid organisms). In cultures from five to six hours old, the charge was low for both organisms. Between nine and ten hours, it rose and later fell slightly. Kahn and Schwarzkopf (1931) reported different phenomena for the tubercle bacillus. In four-day cultures and nine-day cultures, mobility was high, in twenty-day and five-week cultures, much lower. The tubercle bacillus is so different from ordinary bacteria and we know so little about its culture cycle that we cannot expect to interpret the data in analogous terms. There may well be a decrease in the charge on other bacteria in very old cultures. Buggs and Green (1935) indeed reported such a decrease in cultures of *Escherichia coli* and *Staphylococcus aureus* after 127 days; and these observers could find no difference between six-hour and ten-day cultures. Pedlow and Lisse (1936) also reported no change in electrophoretic mobility for *Escherichia coli* between three and twenty-eight hours.

Moyer (1936), in an exhaustive and extremely illuminating study, has given us the clearest and most complete picture of the phenomena involved. He worked with *Escherichia coli* in an aerated medium and demonstrated the following main conclusions. Rough and smooth strains exhibit distinctly different charges but, within each strain, results are highly consistent. Mobility is high at the start and falls during the first hour for the rough strain, remaining low for the second and part of the third hour and then rising again. The mobility of the smooth strain drops during the first and second hours and remains low during

the third and fourth hours. The low mobilities correspond to the lag and beginning of the logarithmic phases, and the subsequent rise in mobility comes toward the end of the logarithmic phase. Moyer made a peculiarly convincing experiment by mixing the large cells from a ninety-minute culture with the small cells from a sixteen- to twenty-four-hour culture when the differential mobility of the two morphological types was clearly manifest under the microscope. The large young cells moved at a mean rate of about  $0.7\mu/\text{sec.}/\text{v.}/\text{cm.}$  while the mean rate for the small old cells was about 0.9.

In a careful analysis of the causative factors involved, Moyer showed that the low mobility of the young cells was not due to irreversible changes in surface caused by buffer; nor to adsorption of dissolved metabolites; nor to adsorption of gaseous metabolites; nor to presence of flagella; but, probably, to a change in physical or chemical nature of the surface of the cells, perhaps associated with "expansion" of the surface and increased permeability. He demonstrated that heating at  $56^\circ$  for  $\frac{3}{4}$  hour did not alter mobility of the 24-hour cells but greatly reduced the mobility of the 3-hour cells.

These results would suggest that all of the previous workers except Sherman and Albus and Pedlow and Lisse, began their observations at too late a period of the culture cycle to detect the phenomena in question. The failure of Pedlow and Lisse, Moyer ascribes to certain technical defects in their procedure, particularly to the use of distilled water as a suspending medium for the electrophoretic determinations.

#### THE PHASE OF LOGARITHMIC INCREASE AND THE COMPLETION OF THE NORMAL CULTURE CYCLE

It has been pointed out in earlier paragraphs that the fifth and final characteristic of "physiological youth" is a rapid rate of cell multiplication. This characteristic (which is the only one observed in ordinary bacteriological studies) is, however, initiated slightly later than the increase in metabolic activity and cell size, the lowered resistance and decreased electrophoretic charge, of the youth phase.

By the time the maximum rate of cell multiplication is reached—at about the middle of the logarithmic phase—most of the other characteristics of youth are on the decline.

Metabolic activity at this period has fallen substantially below its earlier maximum value, as shown by Bayne-Jones and Rhees (1929) and Schmidt and Bayne-Jones (1933) for heat production by *Escherichia coli*, by Eaton (1931) for the respiration of a staphylococcus, by Walker and Winslow (1932), Walker, Winslow, Huntington and Mooney (1934), Mooney and Winslow (1935), and Huntington and Winslow (1937) for production of CO<sub>2</sub> and NH<sub>3</sub> by *Escherichia* and two species of *Salmonella* and by Rahn, Hegarty and Deuel (1938) for fermentation by *Streptococcus lactis*. Schmidt and Bayne-Jones (1933), for example, reported 51 gram-calories  $\times 10^{-11}$  per cell per hour produced in the phase of most rapid reproduction as compared with 232 for the lag period; and Mooney and Winslow (1935) reported  $57 \times 10^{-11}$  mg. of CO<sub>2</sub> per cell per hour at the period of most rapid reproduction against an earlier maximum of 114.

Cell size was noted as reduced at the middle of the logarithmic phase by Jensen (1928), Bayne-Jones and Adolph (1932b), Bayne-Jones and Sandholzer (1933) and Huntington and Winslow (1937) for *Escherichia coli*. This phenomenon did not appear in the studies of Henrici (1928) whose data for *Escherichia coli* and *Bacillus megatherium* show maximum cell size coincident with most rapid reproduction. All the more recent work, however, indicates decreased cell size in this phase. Thus, Bayne-Jones and Adolph (1932b) record a volume of 1 micron just after the point of maximum multiplication rate against 2.5 to 3.5 micra before that maximum rate. Huntington and Winslow (1937), in eight sets of experiments with three *Escherichia* and *Salmonella* species in various media, obtained a mean volume of 0.7 micron for the phase of most rapid cell-division against an earlier mean maximum of 1.1 micron.

Resistance to heat treatment was found by Robertson (1927) to be at a minimum at the height of logarithmic growth (when the "shadow forms" of Jensen, 1928, are most numerous). Elliker and Frazier (1938b) report a survival of under 3 per cent



of colon bacilli heated at 53° when the organisms were in the logarithmic phase as compared with survivals of 34 to 74 per cent for cells from earlier phases of the culture cycle.

The low electrophoretic mobility of the young cells, on the other hand, appears to persist well through the logarithmic phase (Moyer, 1936).

By the end of the logarithmic phase most of the characteristics of physiological youth have wholly disappeared.

Return to pre-lag values for heat-production at the end of the logarithmic phase are reported by Bayne-Jones and Rhees (1929), Mooney and Winslow (1935) and Huntington and Winslow (1937).

TABLE 4

*Milligrams  $\times 10^{-11}$  CO<sub>2</sub> per cubic micron of bacterial substance per hour*

HOURL	PEPTONE	PEPTONE-GLUCOSE
First.....	11	82
Fourth.....	149	132
Tenth.....	17	11
Twenty-fifth.....	12	5

Table 4 from the data presented in the last-cited paper illustrates the general phenomenon, so far as metabolic activity is concerned.

In the peptone medium no cell multiplication had occurred during the first hour and the normal minimum metabolic activity was recorded; in peptone-glucose, however, multiplication began more rapidly and the first hour exhibits the phenomena of physiological youth. By the tenth hour, in both media, reproduction was still going on, though very slowly, and metabolic activity was nearly down to its minimum value.

Cell size was reported as down to its minimal values by the end of the logarithmic phase by Jensen (1928), Henrici (1928), Bayne-Jones and Sandholzer (1933) and Huntington and Winslow (1937). This phenomenon may be illustrated by the data (table 5) from Henrici (1928).

In each case the ten-hour culture was still increasing in numbers but was well past the height of the logarithmic phase.

The recovery at the end of logarithmic increase of normal resistance to harmful agents has been demonstrated clearly by many observers from Schultz and Ritz (1910) and Reichenbach (1911) to Elliker and Frazier (1938b). For example, Schultz and Ritz found that colon bacilli from a 7-hour culture, which had reached its peak population, were completely unaffected by heating to 53° for 25 minutes while a four-hour culture was completely sterilized by the same treatment. The recent data of Elliker and Frazier are almost as striking.

Moyer (1936) finds that at the close of the logarithmic period electrophoretic potential rises again sharply to its normal value.

After the culture cycle has passed through the phase of logarithmic increase, and the phase which Buchanan (1928) describes as that of "negative acceleration" it reaches the "maximum

TABLE 5  
*Size of B. megatherium, micra*

HOURS	STRAIN 1	STRAIN 2	STRAIN 3
0	3.4	5.2	5.2
5	11.3	7.6	9.6
10	4.5	5.0	5.9
20	3.8	3.6	4.4

stationary phase" and then passes through the phases of "accelerated death" and "logarithmic death." With these latter phases we are not concerned in the present review. An optimum cycle, corresponding to the succession of generations in a multicellular organism, is reproduced by transferring to a new medium at the close of the period of active multiplication in the source culture. Such transfer would give us a picture in each new medium of a burst of physiological youth characterized by high metabolic activity, large cell size, low resistance to harmful physical and chemical agents, low electrophoretic mobility and rapid cell division; and, as the culture ages, all these evidences of youth gradually decrease to their initial levels. By transferring at earlier periods, the course of the life-cycle may be "short-circuited," as may be done with mammalian cells in tissue cultures.

The changes in a culture during the phases of accelerated and logarithmic death correspond to the senile changes in the multicellular organism after the normal reproductive period is passed. A comparative study of such senile changes would perhaps be timely; but they represent phenomena of a somewhat distinct type from those apparent in the early phases, which we have considered in the present review.

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# BACTERIAL DISSIMILATION OF CARBOHYDRATES<sup>1</sup>

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Professor J. B. S. Haldane introduces his Sir F. Gowland Hopkins lecture (1937) on the Biochemistry of the Individual with the statement, "The ultimate aim of biochemistry may be stated as a complete account of intermediary metabolism, that is to say, of the transformations undergone by matter in passing through organisms." In this discussion, it is our purpose to reconstruct, in the light of present knowledge, the biochemical events occurring in the living bacterial cell concerned with the dissimilation of carbohydrates. Our knowledge is far from complete; however, "Although it is dangerous to speculate too far, it is foolish not to speculate at all" (Hill).

Our remarks will be more specifically directed toward the bacterial cell although the general problem of cell physiology has received more extensive treatment with yeast and with animal cells, especially those of muscle, brain and liver. There are certain advantages in dealing with metabolic phenomena in highly specialized cells of tissue such as muscle or brain rather than the bacterial cell. In the case of muscle or brain we are dealing with cells functioning in a well-protected and constant environment with respect to pH, temperature, redox conditions, nutritional properties and other factors; whereas, a bacterial cell is virtually a street urchin of the cell world; it is found functioning at temperatures between 80°C. and freezing; at pH levels of less than 1 to above 13; under strict anaerobiosis and strong aerobiosis; with a banquet before it at one time and again subsisting on a

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diet relatively toxic. Accordingly, the bacterial cell is probably endowed with a large variety of enzymes and special mechanisms for use under widely differing conditions.

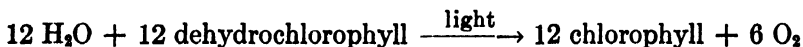
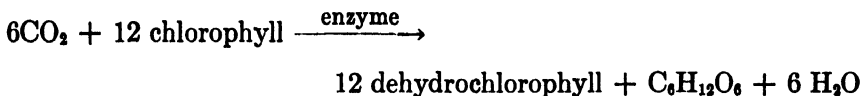
On the other hand, it is not improbable that bacteria offer marked advantages as material for study of the basic physiology of the living cell in its relationships to physical and chemical environment. More particularly those forms referred to as autotrophs show behaviors and characteristics, whose study will doubtless assist in providing an understanding of the primitive and unspecialized cell. According to generally accepted belief of biologists, life on earth is characterized by a gradual though distinct specialization of the simple forms of life to highly differentiated organisms. A spectrum may be visualized showing this transition from organisms requiring only an inorganic substrate for existence, on the one hand, to those extremely parasitic (differentiated) forms such as the viruses on the other. The degree of specialization may be indicated by the adaptability of the organism to its environment. The autotroph must have been among the early forms of life, a conception strengthened by the ease of adaptability to changing environment. In those prehistoric times bacteria must have been chemosynthetic, deriving their energy in the transfer of hydrogen to  $\text{CO}_2$  (oligo-carbophilous forms). It is likely that the use of oxygen is a more recent acquirement inasmuch as aerobes employ the anaerobic hydrogen-activating mechanism in connection with a coordinated aerobic mechanism which permits them to utilize  $\text{O}_2$  as a hydrogen acceptor in place of some intermediately formed product as must an anaerobe. In this sense, an aerobe is an anaerobe which possesses an aerobic mechanism; therefore the anaerobic autotrophs must have preceded the aerobic autotrophs. Anaerobic dissimilation is simpler, less specialized and provides the energy requirements for organisms with low cellular differentiation. *Thiobacillus denitrificans* is an obligate anaerobic autotroph which oxidizes  $\text{H}_2\text{S}$ , S, thiosulfate or tetrathionate to sulfuric acid with the simultaneous reduction of  $\text{CO}_2$  and  $\text{NO}_3$ . The methane bacteria are to be mentioned since some of these forms can obtain their energy chemosynthetically from the process:

$\text{CO}_2 + 4 \text{H}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$ . The better-known aerobic chemosynthetic autotrophs, owing to their utilization of  $\text{O}_2$ , obtain relatively more energy from their substrates. Illustrative of this group are *Thiobacillus thioparus* and *T. oxidans*.

One of the early distinct steps in differentiation occurred with the origin of the photosynthetic forms. When sunlight penetrated to the earth's surface, certain bacteria accepting the possibilities offered by radiant energy used chemical energy to synthesize what was perhaps the first photosynthetic bio-energy transformer. In this case, sunlight furnishes the energy to reduce  $\text{CO}_2$  and during the process  $\text{H}_2\text{S}$  is oxidized to sulfur or its oxides. The existence of photosynthetic bacteria had been suspected since the work of Engelmann (1883, 1888) which described bacteria showing a well defined absorption spectrum. Every effort was made to show the liberation of gaseous oxygen; failing this and recognizing the necessity for  $\text{H}_2\text{S}$ , a chemosynthetic type of metabolism was postulated and gained acceptance in the belief that the dehydrogenation of  $\text{H}_2\text{S}$  provided the energy. Such an explanation, however, did not explain the rôle of light or account for the growth of the bacteria anaerobically. It remained for van Niel (1931, 1935) to reveal the true nature of the process and to harmonize the known facts. He showed that the purple sulfur bacteria oxidize  $\text{H}_2\text{S}$  stoichiometrically to sulfate:  $\text{H}_2\text{S} + 2 \text{CO}_2 + 2 \text{H}_2\text{O} \rightarrow 2 \text{CH}_2\text{O} + \text{H}_2\text{SO}_4$ . In the case of the green bacteria the  $\text{H}_2\text{S}$  is oxidized only so far as sulfur:  $2\text{H}_2\text{S} + \text{CO}_2 \rightarrow \text{CH}_2\text{O} + 2 \text{S} + \text{H}_2\text{O}$ . Roelofsen (1935) demonstrated an endogenous liberation of  $\text{CO}_2$  in the dark; the  $\text{CO}_2$  was immediately assimilated in the light in the presence of  $\text{H}_2\text{S}$ . van Niel has shown that no oxygen is liberated, and that indeed, no liberation of oxygen is to be expected but rather sulfur in the case of the green forms, or its oxides in the case of the purple sulfur bacteria. Members of both the purple sulfur bacteria (*Thiorhodaceae*) and purple bacteria (*Athiorhodaceae*) can utilize molecular hydrogen and certain organic donators.

Photosynthesis appears to show an intimate relationship of chemical and solar energy-yielding processes wherein chemical energy serves to reduce the  $\text{CO}_2$  to carbohydrate and at the same

time oxidize the chlorophyll to dehydrochlorophyll (Conant, Dietz and Kamerling, 1931). Sunlight then furnishes the energy to regenerate the chlorophyll:



The use of sunlight was a great step in relieving the photosynthetic bacteria of chemosynthetic chores; the necessity of providing energy was thus solved by organisms "clever" enough to use chemical energy to synthesize a converter capable of utilizing solar radiation. Since van Niel (1931) has shown that green and purple sulfur bacteria are able to reduce  $\text{CO}_2$  by means of hydrogen from  $\text{H}_2\text{S}$  with the assistance of radiant energy, the development of unicellular chlorophyll-containing organisms would be but a step in phylogenetic development.

The gradation of bacterial metabolism may be further extended. We find the facultative heterotrophic forms which function as autotrophs but can use more complex compounds as sources of either nitrogen or carbon or both, and the facultative autotrophs preferring complex sources of nitrogen and carbon but still able to use inorganic sources. Perhaps these two groups should be recognized as one, i.e., the facultative forms, but the important point is that as the spectrum is extended from the obligate autotrophs to the obligate heterotrophs, the specialization is marked by a gradual and progressive loss of certain synthetic properties. The utilization of  $\text{CO}_2$  by the propionic acid bacteria (Wood and Werkman, 1936, 1938) may represent a vestige of autotrophism in otherwise heterotrophic organisms.

Synthesis of chlorophyll may have been an extremely important event since it would have made free oxygen available as a hydrogen acceptor. That is, it became possible for aerobic forms of life to develop; and the use of free oxygen greatly increased the energy economy of the cell. It is likely that differentiation of the anaerobe into an oxygen-utilizing type led to the

development of series of reversible graded energy systems which released energy for use of the organism in convenient quantities resulting in a smooth, even flow. With increasing differentiation the number of oxidation-reduction systems employed by a cell became greater and successive systems involved more components to yield a smoother flow of energy.

However, differentiation and specialization among the aerobic forms also must have occurred in the course of phylogenetic development. Among the first aerobes were those which used oxygen directly as a hydrogen acceptor. This simplest of aerobic oxidation mechanisms would result in a sudden and uneconomic release of energy. It would be interesting to search for simple oxytropic systems in the primitive bacteria. If such systems do still exist, they must be rapidly disappearing with development of specialization and differentiation. Later, there developed more efficient yet more specialized types of respiration employing hydrogen carriers such as respiratory pigments and flavoproteins, and still later, types such as the cytochrome-containing forms requiring two or more carriers and oxidases.

Certain bacteria require small amounts of a considerable number of growth substances (dissimilation and assimilation factors) in their metabolism exclusive of proteins and carbohydrates. Illustrative of this group are: thiamin (as cocarboxylase) essential in pyruvate metabolism, riboflavin (yellow enzyme group), and nicotinic acid or its amide in hydrogen transportation, adenylic acid and its diphosphate and triphosphate in phosphorylation, vitamin B<sub>6</sub> and pantothenic acid whose functions are unknown at present.

Obviously since the autotrophic organisms do not require the addition of such factors to the medium, they must either synthesize their own or do not require them in their metabolism. Evidence supports the former assumption although instances probably may be found in which the latter assumption applies. *Aerobacter aerogenes* and *Escherichia coli* synthesize thiamin so rapidly that they cannot be depleted by growing on a thiamin-free medium, whereas the more fastidious *Propionibacterium pentosaceum* shows great stimulation on addition of thiamin to cultures

which have been grown on a medium rich in the vitamin and then transferred to a vitamin-deficient medium for not to exceed three successive transfers (Silverman and Werkman, 1938). After additional transfers *Propionibacterium pentosaceum* acquires the ability to synthesize its thiamin. The more parasitic organisms require the addition of the various factors to a synthetic medium, whereas probably many of the factors necessary for growth of the extreme parasites in artificial medium are as yet unknown. The "growth factor" requirements of organisms of this portion of the spectrum probably will be greatly extended.

Bacteria show marked ability to adjust themselves to their environment by selection and by mutation. Dissimilation of specific carbohydrates can no longer be as rigorously accepted in differentiating species or genera as in the past. Bacterial variability reminiscent of a primitive nature is of the greatest value in the study of cell genetics.

Stephenson and her coworkers have demonstrated that adaptation can occur in the parent cell, and it is probably not necessary that the cell multiply in the presence of the specific substrate in order to produce the enzyme for its breakdown. In this case, the addition of the specific substrate to non-proliferating cells is sufficient to evoke the enzyme. In other cases, the presence of the specific substrate has little to do with the appearance of the enzyme which may be effected by other factors. Thus alanine deaminase in *Escherichia coli* is not increased by the presence of alanine and may be all but eliminated by the presence of glucose (Stephenson and Gale, 1937).

It is our experience (Wiggert and Werkman, 1939) that two distinct physiological types of cells of *Propionibacterium pentosaceum* differing in sodium fluoride sensitivity and, more important, in ability to ferment phosphoglyceric acid, result from culturing in the presence and in the absence of sodium fluoride. Bacterial variation must of course be differentiated from effects due to variation in physical and chemical environment during culturing. Since mere traces of elements or compounds may be all that is required for a luxuriant growth or activity, an apparent

variation in a microorganism may result from unintentional differences in the medium.

## DISSIMILATION OF CARBOHYDRATES

For present purposes, bacterial metabolism will be discussed as schematized in figure 1. The discussion will emphasize dissimilation, which may be defined as the transformation of the substrate to yield energy to the organism, as distinguished from those endothermic changes which characterize assimilation. Dis-

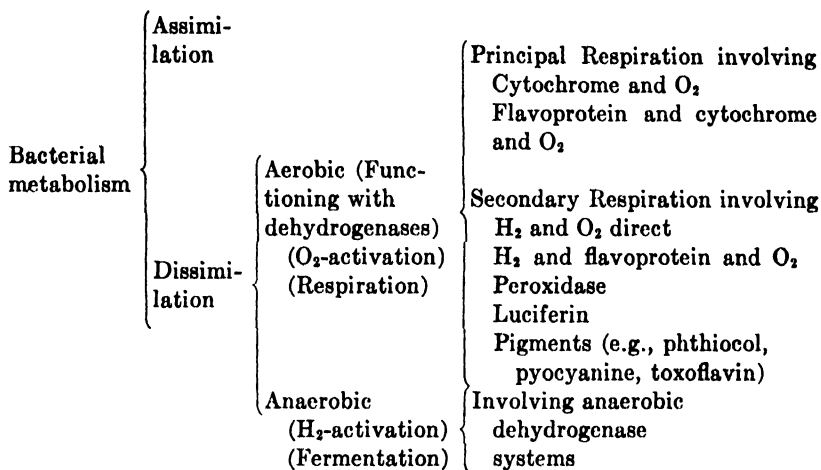


FIG. 1. SCHEMA OF BACTERIAL METABOLISM

similation may, of course, also furnish certain intermediate products necessary as building blocks in assimilation.

For convenience of discussion, we shall speak of aerobic dissimilation and anaerobic dissimilation. The former is respiration, the latter is fermentation in the Pasteurian sense. Both are truly oxidative processes.

We owe much to the genius of Pasteur who recognized that the absence of a respiratory process resulted in "*la fermentation, c'est la vie sans air.*" It was Pasteur who recognized the *physiological equivalence* of fermentation and respiration.

Pasteur (1876) in giving a broad interpretation of what is now known as the Pasteur effect stated (free translation): "Fermenta-



tion is a very general phenomenon. It is life without air, life without free oxygen, or in more general terms, it is the result of a chemical process on a fermentable substance, i.e., capable of producing heat by decomposition."

"Fermentation—a chemical process, connected with the vegetative life of cells—takes place at a moment when these cells, ceasing to have the ability of freely consuming their substrate by respiratory processes—that is, by the absorption of free oxygen—continue to live by utilizing oxygenated substances like sugar. This characteristic (of fermentation) is always ready to manifest itself and in reality does so as soon as life ceases to perform its functions under the influence of free oxygen or without a quantity of that gas sufficient for all acts of nutrition."

Pasteur, with keen insight, has thus given us a concise picture and it is convenient to recognize his differentiation. His general concept has required modification only in one respect in which subsequent investigation has shown lack of breadth, i.e., his insistent demand that the phenomena of fermentation are correlative with life or vital activity (cf. Burk, 1937). Buchner's preparation of an active "press-juice" in 1897 did much to push Pasteur's views into the background for two decades. However, in this respect it should be noted that to date no active preparation, free of cells or cell fragments, of the respiratory enzymes has as yet been obtained. At any rate, the wisdom of Pasteur did not prevent an undue emphasis being placed on the rôle of oxygen in biological oxidation after the discovery of oxygen by Priestley and the enunciations of Lavoisier.

The terms respiration and fermentation have been variously defined and used, even by the same author. It is not so important whether one or another term is used, as it is that the term employed be adequately defined in the light of our present knowledge. Differentiation of the two processes is solely a matter of convenience and no fundamental difference is implied. The intimate relationship of fermentation and respiration is revealed in the Pasteur reaction, a phenomenon which has not been adequately elucidated but does show the quantitative inter-

dependence of the two processes. The linkage between the two reactions has never been discovered although it is generally accepted that aerobic dissimilation is always associated with fermentation, i.e., aerobic carbohydrate dissimilation is always preceded by anaerobic phases (phosphorylation and splitting), in spite of the discovery of enzymes oxidizing glucose directly, or that monoiodoacetic acid at certain concentrations inhibits fermentation and not respiration.

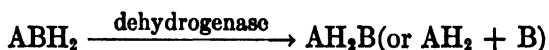
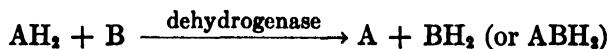
The allocations under Principal and Secondary respiration (fig. 1) are largely speculative and are based on results obtained with cells other than bacteria. They are given here for completeness. Future investigation will show the proper allocations and provide details of mechanism.

### *Biological oxidation*

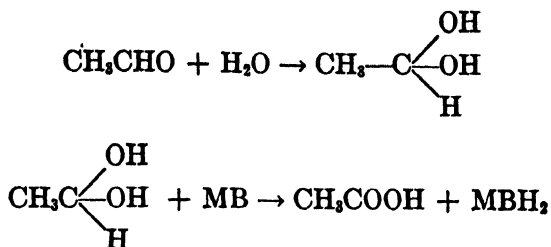
It is now generally accepted that biological oxidation-reduction manifests itself as a transfer of hydrogen (electrons) from donator to an acceptor, the transfer yielding energy to the organism. Clark (1923) has adequately defined biological oxidation as "the withdrawal of electrons from a substance with or without the addition of oxygen or elements analogous to oxygen; or as the withdrawal of electrons with or without the withdrawal of hydrogen or elements analogous to hydrogen." The rôle of oxygen is that of a hydrogen acceptor. Under anaerobic conditions the  $O_2$  is replaced by some other suitable  $H_2$ -acceptor. Thunberg (1917, 1918, 1920) and Wieland (1912, 1913, 1922a, 1922b, 1925) have provided us with the basic concepts of our present knowledge of biological oxidation-reduction.

The transfer of hydrogen is activated by specific enzymes known as dehydrogenases. Wieland's original concept did not provide for the activation of the  $H_2$ -acceptor but only of the donator. It is apparent that certain of the acceptors do not require activation; e.g., methylene blue. On the other hand, probably most naturally occurring acceptors do require activation. The term "activation" has not been defined; nor will any rigid definition be attempted. For the time being, we must sense the mean-

ing. Suffice it to illustrate the point: If succinic acid and methylene blue coexist in solution, no detectable chemical change occurs even after long time, i.e., the substrate is stable even in the presence of oxygen; however, on addition of an active suspension of washed cells of *Escherichia coli*, the methylene blue accepts hydrogen to become reduced methylene blue and succinic acid is dehydrogenated to form fumaric acid. In this case the succinic acid molecule has been activated to donate hydrogen to methylene blue. The trigger-like activation has initiated the the transfer of hydrogen. The term activation is used to express a general concept, i.e., that certain metabolites such as glucose, succinic acid, lactic acid, polyalcohols, etc., are so changed under the influence of certain specific cellular agents that hydrogen atoms may become transferred to reducible substances ( $H_2$ -acceptors), a transfer which otherwise would not occur (Thunberg, 1937). The phenomenon requires a designation, and the term activation is as convenient as any and does not imply the nature of the mechanism responsible (cf. Michaelis, 1933). Not only is activation necessary but the donator and acceptor must be suitable, i.e., the transfer of hydrogen must result in a decrease in the free energy of the system. It should be emphasized that the transfer of hydrogen anaerobically is as truly an oxidation as its transfer aerobically where  $O_2$  is the acceptor. It is now becoming clear that this transfer occurs in orderly fashion and has a complex mechanism. It may occur between molecules or within the same molecule and may be symbolized thus:



Wieland (1925) provided support for his theory by demonstrating the oxidation of acetaldehyde to acetic acid by *Acetobacter* in the absence of free oxygen with methylene blue as the hydrogen acceptor. In this case, the aldehyde is hydrated and two atoms of hydrogen are activated by a dehydrogenase and transferred to the methylene blue.



*Dehydrogenases.* Ehrlich as early as 1885 injected methylene blue into animal tissues and found that most of them reduced the dye. Schardinger in 1902 discovered an enzyme in milk which reduced methylene blue in the presence of an aldehyde. It was not until a decade later that the significance of the action of such enzymes was realized and dehydrogenases were recognized as enzymes activating the release of hydrogen from the molecule of the donator, the hydrogen being transferred to an acceptor. Thunberg's methylene blue technique (cf. Ahlgren, 1936) is generally used to demonstrate the presence of a dehydrogenase. Dehydrogenases may function in the transfer of hydrogen from the donator directly to oxygen as the acceptor. Such dehydrogenases are called oxytropic dehydrogenases and apparently form  $\text{H}_2\text{O}_2$ . It is difficult to differentiate the oxytropic dehydrogenases and the oxidases. Perhaps no harm will result if we think of the dehydrogenases as activating the release of hydrogen, the oxidases as activating the oxygen as an acceptor.

Oxytropic dehydrogenases function with suitable dyes as acceptors in the absence of  $\text{O}_2$  and may, or may not, be cyanide-sensitive. They do not require coenzymes or the cytochrome-oxidase system, and may be considered relatively simple systems in that the activated hydrogen passes directly from the donator to  $\text{O}_2$  without recourse to carrier systems.

The anaerobic dehydrogenases are those capable of activating the release of hydrogen in a system in which the immediate acceptor is not molecular oxygen. Certain of the anaerobic dehydrogenases function through coenzymes which may in turn require a dehydrogenase, e.g., diaphorase, to activate the release of hydrogen from the reduced coenzyme. The route of hydrogen

transfer is generally devious and may not be a single or fixed path; it is not unlikely that any naturally occurring reversibly oxidizable and reducible substance of suitable potential may function as a transporter of hydrogen. Among those occurring in bacteria are riboflavin, cytochrome, coenzyme I and II, pyocyanine, phthiocol and toxoflavin. For a summary of dehydrogenase systems and bacterial fermentations the reviews of Kluyver (1935), Harrison (1935) and Potter (1939) may be consulted.

*Coenzymes.* In addition to the hydrogen donator, the hydrogen acceptor, necessary dehydrogenases and supplementary factors such as water, buffers and inorganic ions, many biological reactions require the presence of coenzymes. A coenzyme may be defined as a dialyzable, thermostable substance necessary in addition to the enzyme and substrate to initiate a reaction. A coenzyme is usually an organic compound although the term was first used by Bertrand (1897) to characterize inorganic ions (Ca and Mn) which activated plant enzymes. The present usage dates from the work of Harden and Young (1905, 1906) who found the thermostable, dialyzable fraction of yeast-juice necessary to initiate fermentation in the residue. Considerable confusion existed regarding the nature of yeast coenzyme (cozymase). In fact any dialyzable, thermostable substance stimulating the action of yeast press-juice was considered a coenzyme; i.e., Mg, K,  $\text{PO}_4$ , hydrogen acceptor (necessary to initiate certain reactions which continue by virtue of acceptors formed subsequently), anti-protease and Euler's principle. Owing to the work of Euler (1936) and Warburg in recent years, remarkable progress has been made in elucidating the function of coenzymes. Cozymase I of Euler and cozymase II of Warburg and Christian, both of which are adenylic acid nucleotides of nicotinic acid amide, are important  $\text{H}_2$ -carriers, capable of passing hydrogen to flavoprotein. Coenzyme II acts with hexosemonophosphate dehydrogenase; coenzyme I acts with hexosediphosphate, lactic acid (muscle), alcohol and malic acid dehydrogenases. Recently Euler and Adler (1938) have shown the biological inter-conversion of the two coenzymes.

A coenzyme may function in one or more of several ways; i.e., as a  $H_2$ -carrier, phosphate-carrier, oxygen-carrier or in ways not now clear. Euler and Myrbäck (1923) proposed the term cozymase for the coenzyme of alcoholic fermentation, whereas the enzyme free from coenzyme has been termed apozymase (Neuberg and Euler, 1931). The present tendency is to use the term coenzyme in referring to thermostable, dialyzable organic substances; e.g., Harden and Young's coenzyme (cozymase, codehydrase, coenzyme I, diphosphopyridine nucleotide), coenzyme II (triphosphopyridine nucleotide), cocarboxylase (thiamin pyrophosphate), and adenylic acid.

Although coenzymes were formerly looked upon as separate entities which accelerated the reactions brought about by the enzymes proper, our point of view is changing. The relationship may involve a union of coenzyme and apoenzyme to form the enzyme (holoenzyme); the coenzyme being a prosthetic group. On the other hand, the coenzyme may constitute a relatively separate entity. In view of recent results, both concepts or a modification may apply. It is probable that in certain cases the coenzyme is very easily dissociated, whereas with other enzymes dissociation does not occur with our present methods. The latter would be represented by those enzymes now considered as not requiring coenzymes. Theorell (1935a) concluded that the dehydrogenase combined with the substrate but not with coenzyme (coenzyme II) although the latter did combine with the substrate-enzyme complex. On the contrary Warburg (1928) insists upon the union of enzyme and coenzyme. Theorell (1935b) split the riboflavin-protein into a protein and a prosthetic group by dialysis. The protein could be combined with flavin phosphate prepared synthetically to form an active enzyme. In a number of cases the protein has been combined with a prosthetic group to yield an active enzyme. It seems that there is ample evidence that it is a question of ease of separation of the holoenzyme (proteid of Warburg) into coenzyme and apoenzyme. As new methods of separation are developed it will be found probably, that enzymes now considered to require no coenzyme will be dissociated into coenzyme and protein carrier.



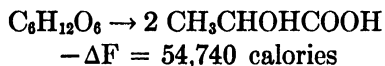
A cell may be able to live an anaerobic existence from the point of view discussed; however, it may have learned also to use molecular oxygen as a hydrogen acceptor.

In figure 2 are illustrated the various types of factors necessary in anaerobic and aerobic dissimilation. The fact must be kept in mind that aerobic dissimilation is conditioned on the presence of suitable dehydrogenase systems.

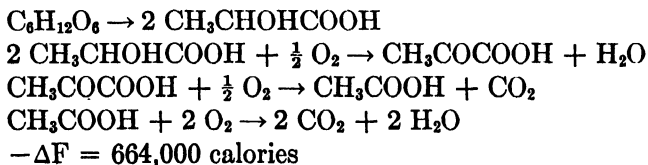
Aerobic dissimilation is more economical than anaerobic dissimilation in the sense that the molal energy of glucose dissimilation by  $O_2$  to  $CO_2$  and  $H_2O$  is approximately a dozen times greater than the anaerobic dissimilation leading only to lactic acid as the final product.

The lactic acid fermentation may serve as an example of anaerobic behavior. We observe that much of the energy represented by the sugar remains locked within the two molecules of lactic acid (cf. Burk, 1937). This process is uneconomical. Now let us assume that the dissimilation occurs in the presence of oxygen as a hydrogen acceptor, and that it results in the formation of  $CO_2$  and  $H_2O$ .

#### Anaerobic Dissimilation Involving Lactic Acid



#### Aerobic Dissimilation to Carbon Dioxide and Water



It should be recalled that the energy released by dissimilation remains constant, so long as the final products remain the same, i.e., the available energy is independent of the path of the intermediary breakdown.

#### *Mechanism of anaerobic dissimilation (fermentation)*

It is convenient to discuss first anaerobic dissimilation and secondly aerobic dissimilation inasmuch as the aerobes appear to have developed from the anaerobes.



Bacterial dissimilation generally involves transformations in the glucose molecule or a polymer as a source of energy. For this reason glucose will serve as the substrate in the discussion of anaerobic dissimilation, and certain analogies will be drawn with schemes of metabolism which have been proposed for muscle and yeast in particular. Recent progress in our knowledge of the biochemistry of muscle metabolism has led to marked changes in our views regarding the dissimilation of carbohydrates by microorganisms, especially the heterotrophic forms.

Our knowledge of the intermediary metabolism of the autotrophic bacteria is quite fragmentary. The metabolism of the heterotrophic and more highly specialized forms bears at least in part, a similarity to that of animal cells with regard to enzymes, coenzymes, carriers and other agents. We have little information regarding the autotrophic intermediary mechanism. Our discussion then is concerned more specifically with bacteria belonging to the heterotrophic *Eubacteriales*.

Numerous theories have been proposed to account for cellular dissimilation; it has been well established that the living cell must have energy to carry on its metabolism, grow and reproduce; this energy is furnished stored in the food molecule which must be rearranged to provide available energy to the cell. This is, of course, excluding the photosynthetic bacteria which utilize the energy of the sun, as do typical chlorophyll-containing plants.

*The Embden-Meyerhof-Parnas theory.* The work of Embden, Deuticke and Kraft (1933) dealt with muscle metabolism. Meyerhof greatly expanded the work with muscle and extended Embden's theory to yeast. The investigations of these workers in the field of muscle and yeast metabolism have proved of inestimable value in the field of bacterial metabolism. Werkman and coworkers have presented experimental evidence that the Embden-Meyerhof-Parnas theory finds application to bacteria by their isolation of the key intermediate of that scheme (phosphoglyceric acid) from a wide variety of bacterial fermentations. Phosphoglyceric acid was first isolated in the case of bacteria from *Citrobacter freundii* (Werkman, Zoellner, Gilman and Reynolds, 1936) and later from *Escherichia*, *Aerobacter*, *Propioni-*

*bacterium* (Stone and Werkman, 1936a, 1936b), and organisms of a relatively large number of genera: *Bacillus*, *Azotobacter*, *Serratia*, *Lactobacillus*, *Streptococcus* and *Staphylococcus* (Stone and Werkman, 1937; Werkman, Stone and Wood, 1937). Recently Endo (1938) in Meyerhof's laboratory has confirmed and extended the work in the case of *E. coli*.

Much of the work has been carried out on cell-free juices of yeast and muscle. Satisfactory bacterial juices are difficult to prepare. Booth and Green (1938) have prepared an active juice in a roller grinder and Wiggert, Silverman, Utter and Werkman (1939) have obtained an active preparation by grinding the cells with powdered glass and centrifuging the resulting juice in a Beams ultracentrifuge. Such an active juice can be dialyzed and essential coenzymes thus removed, studied and identified. In addition to dialysis, specific inhibitors can be used to suppress certain reactions leaving others active; also, certain agents may be used to fix intermediate products for identification. Finally one arrives at results from which a general scheme of fermentation can be synthesized. Silverman and Werkman (unpublished) have prepared a cell-free juice from *Aerobacter aerogenes* capable of making the following conversion:  $2 \text{CH}_3\text{COCOOH} \rightarrow 2 \text{CO}_2 + \text{CH}_3\text{COCHOHCH}_3$ . The simple system may provide a method of attack leading to the elucidation of the synthesis of 4-carbon compounds from 3-carbon substrates. The use of dialyzed cell-free juices should permit us to reconstruct the biochemical events occurring in bacterial dissimilation. This method of attack has proved profitable in studies on animal and yeast metabolism; it should prove equally valuable in studies on bacteria (cf. Parnas, 1938a).

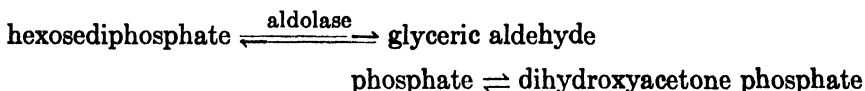
The scheme of Embden, Meyerhof and Parnas is given in figure 3 for reference. The reactions consist of oxidations, hydrolyses and phosphorylations.

It is generally accepted that cellular utilization of glucose involves its phosphorylation with a subsequent split into phosphorylated trioses. Hevesy (1938) and Parnas (1938b) have conducted experiments with tissues, eggs, milk and yeast fermentations with synthetic radioactive adenylic acid, in which it



Phosphorylation of glucose leads to the formation of hexose-6-phosphate which is an equilibrium mixture: glucose-6-phosphate  $\rightleftharpoons$  fructose-6-phosphate. There is as yet no evidence for the formation of the Cori ester (hexose-1-phosphate) (Cori and Cori, 1936, 1937) by bacteria. This ester is formed from the polysaccharides and rapidly converted into the 6-ester. The 6-monophosphate is converted into the Harden-Young ester (fructose-1, 6-diphosphate). Phosphorylation of the hexose leads by an intramolecular rearrangement to an "active" form of the sugar which is indicated to have a furanoid structure by the fact that the cardinal intermediate hexose (fructose-1, 6-diphosphate) is a furanoid. The diphosphate is the precursor of all triosephosphate which reacts with cozymase (coenzyme I). This reduction proceeds slowly (Meyerhof, 1938) unless a phosphate acceptor is present.

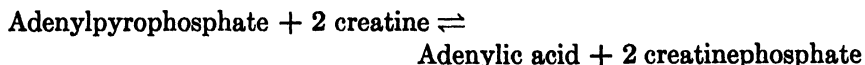
In most tissues the hexosediphosphate is thus converted into a complex equilibrium mixture:



The glyceric aldehyde phosphate is oxidized in a coupled reaction to 3-phosphoglyceric acid  $\rightleftharpoons$  2-phosphoglyceric acid. Initially  $\alpha$ -glycerophosphate is formed by a reduction of a second molecule of triosephosphate. This type of reaction is called dismutation by Neuberg or more generally a Cannizzaro reaction. Subsequently the molecule reduced is not triose but a  $H_2$ -acceptor which forms later in the scheme (e.g., acetaldehyde, pyruvic acid). Enolase changes the phosphoglyceric acid into phosphopyruvic acid which is irreversibly dephosphorylated (Embden, Deuticke and Kraft, 1933) by the adenylic acid system, which may be regarded as a dissociable, active group of the enzyme, phosphorylase. The phosphate is carried by the adenylic acid (adenosinmonophosphate) as adenosindiphosphate or adenosin-triphosphate, both discovered by Lohmann, to glucose which forms initially hexose-monophosphate and then the diphosphate (Harden and Young ester).

Needham and van Heyningen (1935) have reconstructed the

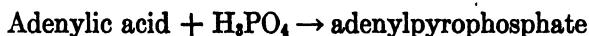
steps between phosphopyruvate and adenylic acid with the formation of adenylypyrophosphate and pyruvate and have shown that dephosphorylation of the phosphopyruvate is exothermic. Nature makes considerable use of phosphate transfer in connecting assimilation and dissimilation. Another reaction in which energy and phosphate are passed between molecules is that occurring in muscle extracts:



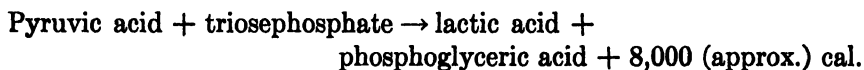
The heat of hydrolysis of adenylypyrophosphate is approximately equal to that required in the synthesis of creatinephosphate.

Creatinephosphate appears to serve as a reservoir for phosphate. It does not occur in yeast, however, and its rôle in bacterial metabolism is uncertain.

It is to be noted that the phosphate appears to work in a closed system although it has been demonstrated with muscle (Parnas and Ostern, 1936; Meyerhof, 1937), yeast (Macfarlane, 1936) and bacteria (Wiggert and Werkman, 1938) that inorganic phosphate is taken up and later released from organic combination. Needham and Pillai (1937) and Meyerhof (1937) have shown the change,



to take place by the energy liberated from the following reaction:



Pyruvic acid appears to be a general intermediary in bacterial dissimilation of glucose. It has been isolated in this laboratory in the case of a relatively large number of genera [*Propionibacterium* (Wood and Werkman, 1934), *Escherichia*, *Citrobacter*, *Aerobacter* (Reynolds, 1935), *Lactobacillus*, *Clostridium*, *Bacillus*, *Azotobacter*, among others (unpublished data)].

In the present state of our knowledge it appears that the Embden-Meyerhof-Parnas scheme of glycolysis finds application in the anaerobic dissimilation of bacteria. However, from experiments with NaF (Werkman, Stone and Wood, 1937; Wiggert

and Werkman, 1939) it is shown that the Embden-Meyerhof-Parnas scheme may not exclusively function in the dissimilation of glucose by *Propionibacterium pentosaceum* but that the organisms may possess in addition some other path of dissimilation. However, there are several facts which support the essential rôle of phosphoglyceric acid as a normal intermediary. First, it is readily isolated; secondly, it is dissimilated to normal final products (cf. Tikka, 1935). A third point is the behavior of other phosphate esters, i.e., phosphoglyceric acid can be isolated from the dissimilation of hexosediphosphate and hence the propionic acid bacteria must possess the enzyme systems requisite for glycolysis by the Embden-Meyerhof-Parnas scheme.

Further investigations may again reveal the greater versatility of bacteria as compared with the more differentiated cells of muscle and brain.

*Dissimilation of pyruvic acid.* Pyruvic acid may be looked upon as a cardinal intermediary in cellular metabolism. It has been shown to occur in the dissimilation of glucose by muscle, brain, kidney, yeast, fungi and bacteria. From it originate many products of cellular dissimilation such as, acetic, butyric, succinic and fumaric acids, ethyl alcohol, glycerol, acetylmethylcarbinol, 2,3-butylene glycol, acetone, isopropyl alcohol, carbon dioxide, and hydrogen. Pyruvic acid may also serve in the formation of amino acids. In muscle glycolysis the pyruvic acid is reduced to form lactic acid; with yeast it is first decarboxylated to acetaldehyde and  $\text{CO}_2$ , the former is then normally reduced to ethyl alcohol although when the fermentation is carried out at relatively alkaline pH levels, a dismutation of the acetaldehyde occurs to form ethyl alcohol and acetic acid in equimolar concentration (Neuberg's type III fermentation). Lipmann (1939) has shown that phosphate must participate in the dehydrogenation of pyruvic acid by lactic acid bacteria and the energy derived can be used to synthesize adenylic acid pyrophosphate from free phosphate and adenylic acid;  $\text{Mg}^{++}$ ,  $\text{Mn}^{++}$  or  $\text{Co}^{++}$  is required. With bacteria the reactions involving pyruvic acid are more complex. Lactic acid (*d*-, *l*-, or *dl*-) may be formed by certain groups, i.e., *Lactobacillus*, *Streptococcus*, *Bacillus*; other groups

possess an essentially alcoholic mechanism, e.g., *Sarcina ventriculi* (Smit, 1930). A mixed lactic-alcoholic type of dissimilation is shown by *Thermobacterium mobile* which converts glucose into lactic acid (about 7 per cent), ethyl alcohol and CO<sub>2</sub> (45 per cent each) (Hoppenbrouwers, 1931). Other bacteria possess more complex mechanisms for the dissimilation of pyruvic acid.

Pyruvic acid may undergo the following changes anaerobically:

- (1)  $\text{CH}_3\text{COCOOH} \rightarrow \text{CH}_3\text{CHO} + \text{CO}_2$  (decarboxylation)
- (2)  $\text{CH}_3\text{COCOOH} + \text{HOH} \rightarrow \text{CH}_3\text{COOH} + \text{HCOOH}$  (hydrolysis)
- (3) (1) followed by:  $2 \text{CH}_3\text{CHO} \begin{matrix} \nearrow \text{CH}_3\text{COOH} \\ \searrow \text{C}_2\text{H}_5\text{OH} \end{matrix}$  (dismutation)
- (4) (1) followed by:  $2 \text{CH}_3\text{CHO} \rightarrow \text{CH}_3\text{COCHOHCH}_3$  (condensation and reduction)  
 $\text{CH}_3\text{COCHOHCH}_3 + 2 \text{H} \rightarrow \text{CH}_3\text{CHOHCHOHCH}_3$
- (5)  $\text{CH}_3\text{COCOOH} + \text{HOH} \rightarrow$   
 $\text{CH}_3\text{COOH} + 2 \text{H} + \text{CO}_2$  (dismutation)  
 $\text{CH}_3\text{COCOOH} + 2 \text{H} \rightarrow \text{CH}_3\text{CHOHCOOH}$

Reaction (1) is shown by both yeast and bacteria (*Sarcina ventriculi*) (Smit, 1930). Reaction (2) occurs apparently with many bacteria especially in the family *Bacteriaceae*. Organisms possessing hydrogenylase split the formic acid into H<sub>2</sub> and CO<sub>2</sub> (*Escherichia coli*) (Stephenson and Stickland, 1932), whereas in other species formic acid accumulates (*Eberthella* type). Reaction (3) is shown by yeast growing in a relatively alkaline medium (Neuberg and Hirsch, 1919), and probably by many bacteria. Reaction (4) may account for the formation of acetylmethylcarbinol and 2,3-butylene glycol by *Aerobacter* and yeast (Neuberg and Reinfürth, 1923). Reaction (5) is a dismutation demonstrated for the heterofermentative lactic acid bacteria by Nelson and Werkman (1936), confirmed by Krebs (1937a) for staphylococci and suggested by Quastel and Stephenson (1925) to account for the anaerobic dissimilation of pyruvic acid by *Escherichia coli*. The propionic acid bacteria bring about a dismutation of pyruvic acid to propionic acid (through lactic acid) and acetic acid and CO<sub>2</sub> (van Niel, 1928; Wood and Werkman, 1934).

Peters (1936) greatly increased our knowledge of the oxidation of pyruvic acid by his studies on the vitamin B<sub>1</sub>-deficient pigeon

brain. The addition of thiamin stimulated the dissimilation of pyruvic acid. Lohmann and Schuster (1937) showed that the coenzyme of pyruvic acid decarboxylation was thiamin pyrophosphate; Lipmann (1937) then showed that thiamin pyrophosphate was involved in the dehydrogenation of pyruvic acid by an acetone preparation of *Bacterium acidificans-longissimum*. Hills (1938) reported a marked stimulation in the pyruvate metabolism of *Staphylococcus aureus* grown in thiamin-deficient media by simple addition of crystalline thiamin. Silverman and Werkman (1938, 1939a, 1939b) have shown that cell suspensions of *Propionibacterium pentosaceum* and *P. peterssonii* require thiamin in the dissimilation of pyruvic acid and that there is an adaptation to the synthesis of thiamin by *P. pentosaceum* after growth in a thiamin-deficient medium. Barron and Lyman (1939) found gonococci, *Streptococcus hemolyticus* and *Staphylococcus aureus* to be stimulated in the breakdown of pyruvic acid by the addition of thiamin.

Although the dissimilation of pyruvic acid has been rather extensively studied, there is every reason to believe that there exist additional ways by which it functions in cellular metabolism of glucose. Wood and Werkman (1938) have suggested in connection with the utilization of carbon dioxide by heterotrophic bacteria that the formation of succinic acid involves pyruvic acid. They showed a direct correlation between the succinic acid formed and carbon dioxide fixed; furthermore, that sodium fluoride inhibits CO<sub>2</sub>-fixation and that the formation of succinic acid is reduced to the same extent. This would suggest the possibility that the utilization of carbon dioxide involves a direct union with pyruvic acid. Perhaps this occurs even in photosynthesis. Should this suggestion be confirmed, it would offer an avenue for direct attack on problems of assimilation.

*Aerobic dissimilation (respiration).* Cellular respiration consists essentially of the dehydrogenation of the substrate molecule with transfer of hydrogen to gaseous oxygen through a series of oxidation-reduction systems involving primarily the transfer of electrons. The energy is thus liberated in a smooth continuous manner. The dehydrogenases adsorb the substrate molecules



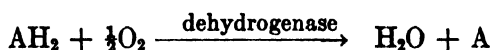
and activate hydrogen which ultimately reaches molecular oxygen. Respiration is frequently defined so as to require the liberation of  $\text{CO}_2$ , i.e., a gaseous exchange. Such a definition cannot be rigidly adhered to since the liberation of  $\text{CO}_2$  is purely incidental. Most respiring cells do evolve  $\text{CO}_2$  although this is not always true. It is desirable to define respiration (aerobic dissimilation) in the sense of a process utilizing molecular oxygen as a hydrogen acceptor. The mechanism of bacterial respiration has received relatively little attention.

In the development of our concepts of respiration, the theory of the activation of the oxygen molecule was first generally accepted following the work of Bach. However, this theory of oxygen activation subsequently was found inadequate in itself. It has been revised by the Warburg school and conciliated with the Wieland theory of hydrogen activation almost simultaneously by Fleisch (1924), Szent-Györgyi (1924), Oppenheimer (1926) and Kluyver (Kluyver and Donker, 1926; Kluyver, 1931), independently. Kluyver particularly emphasizes the far-reaching effects and basic importance of the theory of the unity in the chemistry of cellular metabolism, and points out the general application of the theory of hydrogen transfer in biological oxidation both aerobic and anaerobic.

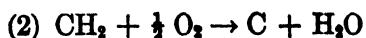
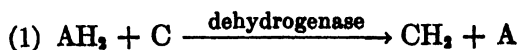
The investigations of Warburg have been concerned mainly with the rôle of iron in cellular respiration. According to his theory, iron in the form of a hematin derivative is intimately associated with the catalysis of respiration; the primary reaction is between iron and molecular oxygen and only in this manner can oxygen be used in respiration. Wieland regarded activation of the hydrogen as of primary importance, whereas Warburg (1928) believed the essential activation was that of the oxygen. Ionic iron was not effective, only iron in a complex organic form (Atmungsferment) was responsible for respiration. It is now known that the Atmungsferment is cytochrome oxidase of Keilin or very closely related. The inhibition of respiration by cyanide is plausibly explained by assuming a union of the iron compound and the cyanide to form an inactive complex. In fact Warburg developed his charcoal model on this premise. When active

charcoal is shaken with oxalic acid, an uptake of oxygen occurs with oxidation of the oxalic acid. The reaction is poisoned by cyanide or urethanes as in the case of living cells. The important point is that Warburg found the similarity between the charcoal model and the living cell lay in the presence of iron in both systems. When charcoals were made from pure cane sugar, the product was free from iron and inactive. Addition of iron salts, however, does not activate the charcoal; in addition organic nitrogen must be added along with the inorganic iron. The investigations of Warburg have done much to explain the utilization of oxygen by the living cell. They have been extended by the brilliant investigations of Keilin.

*Systems of respiration.* The simplest system of respiration will require a hydrogen donator (substrate), a dehydrogenase specific for the donator, and molecular oxygen as the  $H_2$ -acceptor. It is questionable whether organisms depending on such a system exist. Franke and Lorenz (1937) consider their "glucose-oxidase" which oxidizes glucose to gluconic acid to be an oxytropic dehydrogenase. Such a system is illustrated as follows, where  $AH_2$  is the donator and  $O_2$  the acceptor.



This may be the case in the oxidation of amino acids, and aldehydes (Dixon, 1937). Some dehydrogenases not acting directly with  $O_2$  do so through intervention of dyes (e.g., methylene blue) or naturally occurring pigments which require no activation to accept hydrogen and pass it on to oxygen. The dyes that have been used do not occur naturally, although many bacteria contain respiratory pigments, e.g., pyocyanine, phthiocol, chlororaphine and toxoflavin. This type of respiration may be illustrated by the following scheme, where C stands for the hydrogen-carrying dye or pigment.



However, other systems cannot employ  $O_2$  directly as an accep-

tor of hydrogen, although they may do so indirectly; these require specific carriers for the hydrogen. Such naturally occurring carriers themselves may require enzymic activation; e.g., coenzyme I (cozymase) requires activation by diaphorase (coenzyme-factor); flavoprotein requires no such activation. Certain of the carriers function in anaerobic dissimilation, whereas others function in connection with the transfer of hydrogen to molecular oxygen. It is the latter type of carrier in which we are now interested. Moreover, in the same cell, dehydrogenation of different donators may be brought about by different carriers or combinations of carriers. Perhaps the most widely distributed and important of the respiratory hydrogen carriers is cytochrome (Keilin, 1925).

*Cytochrome-cytochrome oxidase system of Keilin.* Largely disregarding the controversy between Wieland and Warburg, Keilin proceeded to conciliate and extend the views of the two investigators. Keilin's rediscovery of cytochrome and his investigations elucidating the properties and behavior of the cytochrome-oxidase system constitute the third in this series of classical investigations dealing with biological oxidation-reduction.

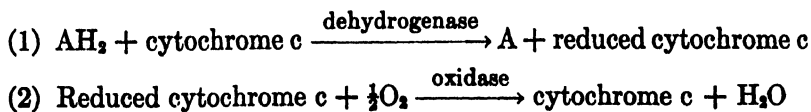
MacMunn in 1886, while investigating absorption spectra of tissues, described absorption bands of hematin compounds which are now known to have been those of cytochrome. Severe criticisms came, however, from Hoppe-Seyler (1890) and the initial discovery of cytochrome (called myo-hematin and histo-hematin by MacMunn) fell into disrepute and was not accepted until Keilin in 1925, rediscovered and named the respiratory haemochromogen, cytochrome.

Before discussing the rôle of cytochrome in bacterial respiration, it is desirable to make a few general remarks regarding cytochrome and its function in cellular respiration.

When a heavy suspension of aerobic or facultative bacteria under anaerobic conditions is examined spectroscopically, the four bands of cytochrome are plainly visible. Keilin regards cytochrome as consisting of three separate hemochromogens, cytochromes a, b, and c, each with two bands. Three of the bands (one from each of the three cytochromes) constitute nearly coin-

cident lines and comprise band d. Bands a, b and c are separate and belong to cytochromes a, b and c respectively. Cytochrome is a hemochromogen, i.e., it is a reduced hematin combined with a protein or other organic nitrogenous group. The important point is that cytochrome is oxidized and reduced in a living cell; it acts as a hydrogen carrier, passing the hydrogen on to molecular oxygen which has been activated by an enzyme formerly called indophenol oxidase and now known as cytochrome oxidase. Apparently, the union of hydrogen with oxygen is unusual in that no peroxide is formed. With respect to oxidation and reduction, the essential part of the cytochrome molecule is the iron which can undergo oxidation from the  $\text{Fe}^{++}$  to the  $\text{Fe}^{+++}$  state. It is not pertinent at this point whether the oxygen unites with the cytochrome, perhaps it is sufficient to say that water is formed in the reduction of the oxygen by hydrogen present. The transfer of an electron from  $\text{Fe}^{++}$  to  $\text{H}^+$  results in  $\text{Fe}^{+++}$  and  $\text{H}$  (represented by oxidized cytochrome and water). Succinic acid is the principal donator (cf. Szent-Györgyi, 1937) in animal tissue, although it has recently been shown that hexose-monophosphate may function. Our knowledge of bacterial respiration is still too fragmentary to speculate on the occurrence of events in bacteria although succinic acid is of widespread occurrence and there is evidence of stimulation of washed cells by the addition of the succinic-fumaric acid system. Cytochrome is of general occurrence among aerobic and facultative bacteria (Tamiya and Yamagutchi, 1933; Yaoi and Tamiya, 1928; Yamagutchi, 1937; Frei, Riedmuller and Almasy, 1934) and absent from most if not all strict anaerobes. Such facultative organisms as the propionic acid bacteria contain the cytochrome-oxidase system and it appears probable that continuous culture of such forms in the presence of oxygen leads to an increased activity of the cytochrome-oxidase system.

The function of cytochrome may be illustrated as follows:



The reduced form of cytochrome c unlike other hemochromogens is not oxidized spontaneously by oxygen but requires the action of a specific enzyme, cytochrome oxidase. The absorption spectrum of reduced cytochrome may be clearly distinguished in active cultures of bacteria, even in such essentially anaerobic forms as the propionic acid bacteria.

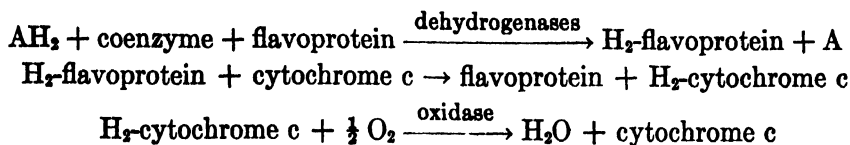
In remaining types of respiration the hydrogen may be transferred through two or more carriers. Such systems may involve flavoprotein, i.e., "yellow enzyme" of Warburg. Warburg's (cf. Warburg and Christian, 1938) terminology has been criticised by Thunberg (1935) and Ogston and Green (1935a). For a discussion and comparative table of nomenclature see Dixon (1939).

*Flavoprotein.* Flavoprotein comprises a protein carrier with a prosthetic group discovered by Banga and Szent-Györgyi (1932) who called it cytoflav (cf. Laki, 1933) and suggested that it played a rôle in respiration. Our knowledge of the action of flavoprotein is due largely to the work of Warburg and Christian (1933) who isolated the conjugated protein from yeast and showed that the prosthetic group is lactoflavin-5-phosphoric acid (also called riboflavin phosphate or alloxazine nucleotide) and that it does not function efficiently as a carrier until combined with a protein. The active nucleus of the prosthetic flavinphosphate group is iso-alloxazine. Flavin is iso-alloxazine combined with ribose and is commonly called riboflavin.

Kuhn, Rudý and Weygand (1936) synthesized the prosthetic group; and by combining it with Theorell's protein (1935b) Kuhn and Rudy (1936a, 1936b) showed that the complex was active.

Reduced flavoprotein is spontaneously oxidized by molecular oxygen when no other electroactive system of higher potential is present (cf. Warburg and Christian, 1933). Normally it transmits its hydrogen to oxygen by way of cytochrome c when present, since the latter step is much more rapid at the lowered oxygen tensions presumably present in the cells (Theorell, 1936). The flavoprotein functions with specific dehydrogenases which require hydrogen carriers between the substrate and the flavoprotein.

According to Ogston and Green (1935a), flavoprotein reacts with greatest activity, as a hydrogen carrier, with hexose diphosphate, hexosemonophosphate, glucose and malate as donators. The normal behavior of flavoprotein may be represented:



In the presence of cyanide inhibition of the cytochrome oxidase, flavoprotein may perhaps, transmit hydrogen directly to oxygen although Theorell considers such a transfer as unphysiological. Since *L. delbrueckii* contains no cytochrome which can serve to accept hydrogen from the flavoprotein, its reoxidation probably depends on anaerobic hydrogen acceptors in the case of these bacteria. Flavoprotein appears to act as a hydrogen carrier between slowly reacting systems and its rôle in anaerobic dissimilation is probably that of a carrier. Coenzymes I and II reduce it. Since riboflavin phosphate can be reversibly oxidized and reduced, it behaves as an indicator.

Although previously only one flavoprotein was known, recently new ones have been discovered in which the prosthetic group is flavin-adenine-dinucleotide (coenzyme of amino acid oxidase). Haas (1938) has isolated a second flavoprotein from yeast in which the prosthetic group, like that in milk flavoprotein, is flavin-adenine-dinucleotide and the protein carrier differs from that of the original yeast flavoprotein and with which lactoflavin-phosphate is inactive. Haas obtained both fractions separately and was able to combine them to form the active flavoprotein.

Diaphorase, the dehydrogenase of coenzyme I, recently isolated by Straub (1939) and Straub, Corran and Green (1939) is a flavoprotein. The presence in animal tissue of an enzyme catalyzing the oxidation of reduced coenzymes I and II was demonstrated by Adler, Das and Euler (1937) and Dewan and Green (1938). The latter have demonstrated the presence of diaphorase in *E. coli*, *Bacillus subtilis* and *Bacterium proteus*.

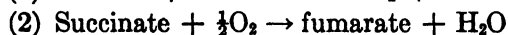
Flavoprotein has been spectroscopically estimated in a number of genera, e.g., *Lactobacillus*, *Acetobacter*, *Clostridium*, *Flavobacterium*, *Escherichia* (Schütz and Theorell, 1938). Its occurrence in cells is general. Schütz and Theorell (1938) observed that *Lactobacillus delbrueckii*, *E. coli*, *Streptococcus lactis* and *Flavobacterium lacticum* did not alter their flavoprotein content when subjected to aerobic and anaerobic conditions, a finding which supports the view of Theorell (1936) that physiologically flavoprotein does not react directly with oxygen.

Wood, Andersen and Werkman (1938) have shown riboflavin to stimulate growth of *Lactobacillus* and *Propionibacterium* when added to a medium deficient in this constituent. Doudoroff (1938-39) has shown a similar stimulation of luminescent bacteria.

The investigations of Szent-Györgyi (1937) and of Krebs (1937b) have led to recognition of still another type of respiration based on fumaric acid catalysis. In the work of Szent-Györgyi, it was found that succinic acid was rapidly dehydrogenated to fumaric acid through cytochrome. These investigators believe that fumaric acid is a  $H_2$ -carrier along with oxalacetic acid. According to Krebs, fumaric acid is a carrier in the respiration of certain bacteria such as *Escherichia coli* and *Staphylococcus aureus*.

If fumaric acid is a carrier, it should, if present in sufficient quantity, carry on the oxidation of the substrate in the absence of oxygen and be recovered as succinic acid; and the rate of reaction should not be less than that in the presence of free oxygen. Furthermore, it must be shown that succinic acid occurs under physiological conditions in the dissimilation of the substrate and is oxidizable in the presence of oxygen.

The rate of the reaction can usually be determined by measuring  $CO_2$  evolved or substrate disappearing. The reactions may be set up as follows:



Reaction 1 must occur as rapidly as number 2.

Table 1 taken from Krebs (1937b) illustrates the point.

The final step is to show that the succinate formed will transport hydrogen to  $O_2$  as rapidly as glucose since no reaction can

limit the rate to less than that of glucose. In fact, the rate of transport of hydrogen to oxygen from succinate is greater than the rate of glucose breakdown. Since this is true, some other reaction must limit the breakdown of glucose.

An objection which has been raised against the theory of Szent-Györgyi is based on the well known fact that the 4-carbon dicarboxylic acids are intermediary products in carbohydrate metabolism and are burned in the cell, the inference being that irre-

TABLE 1

*Rates of oxidation of glucose by *Bacterium coli* by molecular O<sub>2</sub> and by fumarate (Krebs, 1937b)*

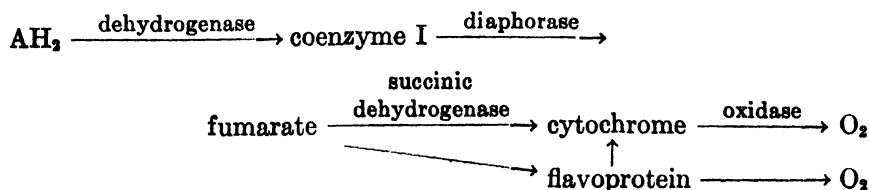
40°; pH 6.8; 2 mg. (dry weight) bacteria per flask.

TIME AFTER THE ADDITION OF 0.507 MGM. GLUCOSE	CO <sub>2</sub> PRODUCED (μl.)	
	In the presence of O <sub>2</sub>	Anaerobically in the presence of M/20 fumarate
<i>minutes</i>		
10	60.5	78
20	139	162
40	219	198
60	237	223
80	246	245
100	254	262
120	263	280
Succinic acid* found in the solution (μl.).		510

\* One millimol = 22,400 μl.

versibly oxidized substances cannot function as catalysts. However, we have found a catalytic effect in the metabolism of *Micrococcus lysodeikticus* at low concentrations of fumaric acid. The increase in oxygen taken up exceeded by several times the quantity required to oxidize the fumaric acid added.

The succinate is reoxidized to fumarate by the cytochrome-oxidase system. The type may be represented:





Although cell respiration is the sum total of oxygen used by all types present in a cell, there is practically no knowledge of the proportional respiration accounted for by the different mechanisms.

#### PRINCIPAL AND SECONDARY RESPIRATION

From the standpoint of the cell, respiration is frequently classified either as (1) principal and secondary or (2) cyanide-sensitive and cyanide-insensitive. Principal respiration (Hauptatmung) is that constituting the main types in a normal cell, whereas secondary respiration (Nebenatmung) is that which constitutes relatively incidental (non-ferment hemin) respiration. In the main, principal respiration is that due to ferment-hemin (cytochrome) and is cyanide-sensitive although part of the secondary is also sensitive. The principal respiration probably includes certain reactions such as that due to flavoprotein which normally brings about oxidation through the cytochrome-oxidase system but which may transmit hydrogen directly to oxygen when the cytochrome is poisoned by cyanide. Alt (1930) has shown that cyanide-insensitive respiration is less in normal cells than in those that have been injured during the experiment. It is frequently claimed that this observation proves the abnormal character of such secondary respiration. The finding of Gourévitch (1937) that the amount of extractable flavin is proportional to cyanide-insensitive respiration indicates that this secondary respiration is due to a change in the behavior of the "yellow respiratory enzyme" to react directly with oxygen after inhibition by cyanide. It is probable that a substantial part of the secondary respiration of bacteria consists of pigment respiration. Present in bacteria are many pigments which function as carriers or acceptors, accepting hydrogen and passing it on perhaps directly or indirectly to molecular oxygen. Among the better known are pyocyanine, phthiocol and toxoflavin not to mention those such as the flavins which have been more thoroughly discussed already. Pyocyanine according to Friedheim (1931) strongly stimulates the respiration of pigment-free strains of *Pseudomonas aeruginosa* as well as of red blood cells. He found a more marked effect on

cells containing hemin systems. Such catalysis is cyanide-sensitive. Reduced pyocyanine is autoxidizable and in those cases where the oxidation is brought about by direct reaction with molecular oxygen (without iron) cyanide will exert no effect; e.g., pyocyanine respiration of the anaerobic tetanus bacteria (Frei, 1934). When pyocyanine acts as the  $H_2$ -acceptor in the respiration of bottom yeast with hexosemonophosphate as the substrate, cyanide (M/600) causes an inhibition of 31 per cent of the pyocyanine catalysis (Ogston and Green, 1935a, 1935b). The respiration of *Staphylococcus aureus* is strongly stimulated by pyocyanine (Ehrismann, 1934).

Phthiocol is a yellow pigment found in the tubercle bacillus. It exists in the bacteria in oxidized form and is a relatively negative redox system. Its effect on respiration has not been reported. *Bacterium violaceus* contains a pigment, violacein, which increases respiration of bacteria freed of their pigment. Since the reduced form is not autoxidizable, it must function as a hydrogen carrier according to Friedheim (1932). Chlororaphin, the green pigment of *Bact. chlororaphis* forms a reversible system sharing many characteristics with pyocyanine (Elema, 1933). Toxoflavin found in *Bact. bongkreke* (van Veen and Mertens, 1934) is the prosthetic group of a toxic yellow pigment and is a reversible system, electroactive between pH 4 to 8 (Stern, 1935). Phoenicein found in *Penicillium phoeniceum*, is a reversible redox system. The reduced form is autoxidizable. The fungus contains no cytochrome and its respiration may be iron-free. Phoenicein increases the respiration of *Ps. aeruginosa* several times (Friedheim, 1933). In addition, secondary oxidations by  $H_2O_2$  with peroxidases and simple iron systems involving direct attack on the substrate may occur. These would be part of the secondary respiration but cyanide-sensitive, since peroxidases have been shown to contain iron. Finally, as part of secondary respiration there may occur a direct oxidation of unsaturated linkages especially in the fatty acids by hemin (Kuhn and Brann, 1927) or metal salts (Rosenthal and Voegtlin, 1933).

Haas (1934) has given us evidence in the case of yeast, regarding the relative intensities of the principal and secondary respirations.

Using a spectroscopic method, Haas demonstrated that in the intact cells practically all of the oxygen consumption is due to the activity of the cytochrome system.

Roman (1938) lists under "Nebenatmung"

A. Dehydrogenations

- (1) Oxytropic dehydrogenation (Schardinger reaction).
- (2) Flavoprotein
- (3) Oxyhydrogenases and oxidases: oxidative deamination of amino acids, protamines, tyrosine and uric acid.
- (4) Quinone catalyses by autoxidizable chromogens.
- (5) Secondary oxidations by  $H_2O_2$ .
  - (a) Direct on the substrate.
  - (b) By peroxidases and thermostable iron systems (pseudo-peroxidases).

B. Non-dehydrogenation (peroxide) oxidation as with unsaturated fatty acids; perhaps further little-known mechanisms, i.e.,  $\omega$ -oxidation and ring splitting (proline).

Studies on cellular metabolism have confirmed the view, originally proposed by Wieland and conciliated with the view of Warburg, especially by Kluyver, that the essential principle of cellular metabolism involves the transference of hydrogen from donator to acceptor. A series of graduated redox systems provides the mechanism of transfer and results in a smooth and regulated flow of energy for the use of the cell in assimilation. Under anaerobiosis, the ultimate transfer is limited to reducible intermediate products of dissimilation; under aerobiosis, oxygen is the final acceptor. Here the systems of Warburg and Keilin are active. Hydrogen, activated by suitable dehydrogenases and transported by carrier systems, is accepted by the inert oxygen only after activation of the oxygen molecule. This is accomplished by alternative reduction of the  $Fe^{+++}$  in the prosthetic group of hemin compounds by the hydrogen, and oxidation of the  $Fe^{++}$  by the oxygen from the air. This path must constitute, in the main, that of the principal respiration; in addition a certain uptake of oxygen occurs in the absence of the hemin compounds. Our knowledge of bacterial respiration is too inadequate to reconstruct the respiratory systems with any degree of assurance. In

the light of our present knowledge, it appears that the principles and the basic mechanisms underlying bacterial dissimilation are substantially those found in the higher, differentiated forms of life but providing for greater adaptation and variation.

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